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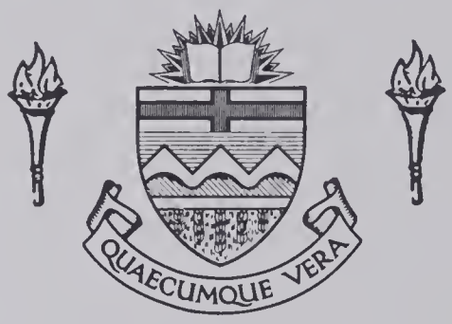

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DRUG STABILIZATION OF EXCITABLE MEMBRANES

by

RICHARD S. NEUMAN

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Drug Stabilization of Excitable Membranes , submitted by Richard S. Neuman in partial fulfilment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

Voltage-clamp experiments were carried out on single myelinated nerve in order to investigate the stabilizing action of various drugs. Experimental results were analyzed in terms of the Hodgkin and Huxley model of nerve excitability.

Drugs that are usually classified as general cellular depressants such as phenobarbital, pentobarbital, chlorpromazine, diphenhydramine and procaine, were found to block excitability by reducing the maximum sodium conductance with or without effects on the potassium conductance or the sodium or potassium kinetic parameters. Chloral hydrate and trichloroethanol on the other hand block excitability as a result of both reduction in the maximum sodium conductance and altering the voltage dependence of the sodium system kinetics.

Further experiments were conducted with some of the above drugs to examine the possibility that altering the external calcium concentration would modify their depressant effects on the membrane conductances. There was some indication that chlorpromazine was more effective in reducing the sodium and potassium conductances when the extracellular calcium concentration was reduced whereas the actions of phenobarbital, pentobarbital and

trichloroethanol were modified only slightly or not at all.

Drugs useful in the treatment of grand mal epilepsy such as phenobarbital and diphenylhydantoin were found to stabilize the nodal membrane against hyperexcitability by an action on the kinetic parameters of the sodium system.

The action of one 'labilizer' (strychnine) was also studied. Strychnine was found to prolong the action potential and produce potassium inactivation.

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THE IONIC BASIS OF EXCITABILITY IN FROG MYELINATED NERVE FIBRES

In 1952 Hodgkin, Huxley and Katz published a series of five papers describing the ionic basis of excitability in giant axon from Loligo (Hodgkin and Huxley, 1952 a, b, c, d; Hodgkin, Huxley, and Katz, 1952). These papers gave a quantitative description of much of what had been found from classical studies on excitability in both nerve and muscle. More importantly however, they laid the foundation for the "sodium hypothesis" and thereby opened a new era in membrane biophysics by demonstrating that it was the flow cf sodium icns which produced the action potential and not simply a nonspecific increase in membrane permeability tc all ions as was originally proposed by Berstein in 1905 (Cole, 1968). It was the development of the voltage clamp technique by Cole (1949) and its improvement by Hodgkin, Huxley and Katz (1949) that allowed Hodgkin and Huxley to dissociate the components of the excitability process in nerve and examine them in detail. It is appropriate then that the discussion begin with a brief description of the principles of the voltage clamp technique and the results obtained.

Voltage Clamp Principles

Essentially the voltage clamp technique allows the potential across a patch of membrane to be measured and under control at all times. Cole (1949) accomplished this control in the squid axon by inserting an axial wire into the axoplasm. The potential difference between the internal electrode and an external electrode across a small patch of membrane could then be measured and compared to a command voltage at the input of a feedback amplifier. Any difference between the command voltage and the membrane voltage forces the feedback amplifier to add or subtract current from the membrane to maintain the potential difference across the membrane equal to the command voltage. The current which flows in the feedback amplifier is then a measure of the current which flows through the membrane at that particular voltage. If a voltage step is applied at the command input, the membrane will be forced to follow the voltage step, and the currents which flow while the membrane is reaching a new equilibrium can be measured. In principle the voltage clamp technique is similar to temperature jump techniques where the temperature of an enzyme system or chemical reaction mixture may be suddenly changed and the relaxation kinetics of the process studied.

The voltage clamp method is an important advance in technique for two reasons. First, it allows measurement of the current flowing across the membrane. Since the nerve fibre may be treated as a leaky cable, the Kelvin cable equation for current flow which varies with time and potential may be given by

$$i_m = C_m \partial V/\partial t + V/r_m = [\ 1/(r_e + r_i)\ ]\ (\partial^2 V/\partial x^2) \qquad (1)$$

where i_m is the radial membrane current in amperes/cm, C_m is the membrane capacity in farads/cm, r_e and r_i are the external and internal longitudinal resistances respectively in ohm/cm, x is the distance along the cable in cm, V is the potential difference across the membrane in volts, t is the time in seconds, and r_m is the membrane resistance in ohm cm. Since the action potential is a disturbance that varies in potential and time as it passes down a nerve fibre, it can be seen that a solution to the current flow would be extremely difficult. When a nerve is voltage clamped however, there is no voltage gradient along the fibre over a short distance so the equation reduces to the simple differential equation:

$$i_m = C_m\ dV/dt + V/r_m \qquad (2)$$

Further, since after a step of potential $dV/dt = 0$

the equation reduces to,

$$i_m = V/r_m = i_i \qquad (3)$$

where i_i is the ionic current flowing through the membrane in amperes/cm.

The second reason of importance is that membrane conductances have been fcund to be a function of time and of the potential across the membrane but not current. That is, when the membrane is under potential control and the potential is changed, the currents produced show a graded response. However, if the membrane is under current control (current clamp) and the current is changed in such a way that the membrane is depolarized, then a point is reached where the membrane produces an ungraded action potential which is not turned off by reducing the current to zero. In other words the membrane is only stable under potential control.

In principle the voltage clamp methods for the squid axon and the nodes of Ranvier are similar; in practice it is more difficult to clamp the node because the diameter of the nerve fibre is so small one must find other ways than an axial electrode to achieve potential control across the membrane (Moore and Cole, 1963; Dodge and Frankenhaeuser, 1958; Nonner, 1969).

The Nature of the Conductance Changes Underlying Excitation

It is very convenient to divide excitation into two parts; 1) the ions and their driving forces which are responsible for the currents and 2) the kinetics of the ion movement (Noble, 1972). The reason for the division is that the two processes may react very differently to various drugs and ions which may be added to the inside or the outside of the axon. For example, tetrodotoxin can abolish the movement of sodium ions through the membrane and yet leaves the kinetics of the "sodium channel" unaffected (Hille, 1967a, 1968a; Kao, 1966; Narahashi, 1971), whereas nickel may have marginal effects on the sodium conductance mechanism and yet prolong the kinetics of both sodium activation and inactivation (Dodge, 1961).

The concept that sodium and potassium ions flow through separate "channels" will be used throughout this thesis. Hille (1967a, 1970), has discussed the evidence for both the concept of a "channel" and the seperation of the sodium and potassium "channels". The Q_{10} values of 1.3 for both the sodium and potassium conductances is similar to that for electrolytes in aqueous solution suggesting that the ions move through the membrane by diffusion in an aqueous medium. Further, the number of

ions moved and their direction is a function of the driving force on the ion and does not show any evidence of saturation which is what would be expected with a carrier-mediated process.

Hodgkin and Huxley found in the squid axon that, in general, the membrane behaves like an ohmic conductor. That is,

$$i_m = g_m \triangle E \qquad (4)$$

where g_m is the conductance of the membrane in mho/cm and $\triangle E$ is the driving force on the ions expressed in volts. g_m may be thought of as the ease with which the ions may pass through the membrane and is therefore related to the permeability of the membrane to that particular ion (Hope, 1971). Since the ions that pass through the membrane are charged, it seems obvious that the membrane potential will influence the direction and magnitude of the ion flow. In addition, since there is a potential for each ion at which no net current will flow, one would expect that the driving force on the ion would be equal to the difference between the membrane potential and the equilibrium potential for that ion. The equilibrium potential Er is given by the Nernst equation

which for a monovalent cation would be as follows,

$$Er = (RT/F) \ln [X]e/[X]i \qquad (5)$$

where R is the gas constant in joules, T is the absolute temperature in degrees Kelvin, F is the Faraday constant in coulombs/equivalent, and [X]e and [X]i are the external and internal concentrations of the ions considered respectively (strictly speaking these latter values should be expressed as activities).

In frog nodes and in squid axons, the major currents are the sodium, potassium, and leakage currents. For the squid axons the equations for the currents are given by

$$I_{Na} = g_{Na}(E - E_{Na}) \qquad (6)$$

$$I_K = g_K(E - E_K) \qquad (7)$$

$$I_L = g_L(E - E_L) \qquad (8)$$

where E is the membrane potential given by the difference between the inside and the outside potential, and E_{Na}, E_K, and E_L are the equilibrium potentials for sodium, potassium, and the leakage ions. These equations are justified because the infinite (instantaneous) frequency conductances were found to be linear. That is when the

membrane was stepped to a new potential, the conductance for each ion corresponding to that potential was reached immediately, and the current for each ion was ohmically related to the conductance and driving force for that particular ionic species.

In nodes from frog and toad the instantaneous relationships between sodium and potassium currents and voltages were not linear. The leakage current however, was found to be linear. That is, the nodal membranes show rectification. For the same membrane conductance the current is larger when the ions move from a higher to a lower concentration than in the reverse direction. The "constant field equation" (Goldman, 1943) gave a reasonable fit to the data and was therefore used to describe the sodium and potassium permeabilities. If we let X stand for sodium or potassium, then the currents are given by

$$Ix = Px\,[X]e\,(F^2E/RT)\,[\,(\exp[\,(E - Ex)\,F/RT\,] - 1)/(\exp[\,EF/RT\,] - 1)\,] \qquad (9)$$

where Px is the permeability coefficient given by

$$Px = Ux\,RT/\delta \qquad (10)$$

where Ux is the mobility of the ion X and δ is the

membrane thickness (Dodge and Frankenhaeuser, 1959; Dodge, 1963). Because the leakage conductance was constant, the leakage current I_L was given by equation (8).

The Kinetics of the Current Changes

In the discussions of the mathematical model of the current changes which follow, it should be understood that this model is wholly empirical. Without any knowledge of the structure of the membrane or how the ions actually pass through the membrane, Hodgkin and Huxley decided on a model which they hoped might have a physical explanation, but had no guarantee that it did; nor do we today. Other equations could have been fitted to the data and the ones that were fitted have not gone unchallenged (Hoyt, 1963; Hoyt and Strieb, 1971; Goldman and Schauf, 1972). In fact, the basic concept that the membrane is the control site for cellular permeability has been challenged by Ling (1962) who postulated an association-induction hypothesis, and by a number of Soviet scientists (Troslin, 1966). Nevertheless the model has proved extremely powerful in predicting nerve behavior and in analyzing drug action on nerve. Both the frog node analysis of Dodge (1963) and the toad node analysis of Frankenhaeuser (1960, 1963) follow the Hodgkin and Huxley analysis quite closely.

Dodge chose to describe the time and voltage

dependent sodium and pctassium conductances by three dimensionless variables, m, h, and n, all of which could assume values between zero and one in a manner similar to those employed in the squid model. n represents the fracticn of the potassium system which is activated, whereas h describes that fraction of the sodium system available for activation, and m that available fraction of the sodium system that is activated. 1-h is known as sodium inactivation. The sodium current is more complex than the potassium current because with a sustained depolarization the sodium current first rises and then falls, while the potassium current turns on and remains on. Each of the parameters was assumed to obey first order kinetics so,

$$dn/dt=A_n(1-n) - B_n n \quad (11)$$

$$dm/dt=A_m(1-m) - B_m m \quad (12)$$

$$dh/dt=A_h(1-h) - B_h h \quad (13)$$

where A and B represent the respective forward and backward rate constants which are voltage dependent. The steady state values are given by:

$$\underline{n}=A_n/(A_n+B_n) \quad (14)$$

$$\underline{m}=A_m/(A_m+B_m) \tag{15}$$

$$\underline{h}=A_h/(A_h+B_h) \tag{16}$$

After a step change in voltage the solutions to the rate equations are,

$$n=\underline{n}-(\underline{n}-n_o)\ \exp\ [-t/T_n] \tag{17}$$

$$m=\underline{m}-(\underline{m}-m_o)\ \exp\ [-t/T_m] \tag{18}$$

$$h=\underline{h}-(\underline{h}-h_o)\ \exp\ [-t/T_h] \tag{19}$$

where n_o, m_o, and h_o, are the initial values. The time constants T_n, T_m, and T_h are given by:

$$T_n=1/(A_n+B_n) \tag{20}$$

$$T_m=1/(A_m+B_m) \tag{21}$$

$$T_h=1/(A_h+B_h) \tag{22}$$

When the nodal membrane is depolarized there is some delay before the scdium current turns on, but when this current turns off the fall is adequately described by a single exponential term. To account for this delay Dodge

used the same simplification as Hodgkin and Huxley and empirically raised m to the third power. Frankenhaeuser found that in the toad m^2 was a better approximation than m^3. The sodium permeability in the frog node may then be described as

$$P_{Na}=\bar{P}_{Na}\, m^3 h \qquad (23)$$

where $\bar{P}_{Na}$ is the maximum sodium permeability. This equation is identical to that for the squid model except that in the latter conductance is substituted for permeability.

In the potassium system there is even a longer delay than with the sodium system so that n was raised to the fourth power. The potassium conductance was therefore given by,

$$g_K=\bar{g}_K n^4 \qquad (24)$$

where $\bar{g}_K$ is the maximum potassium conductance. Although $\bar{P}_K$ should have been used in place of $\bar{g}_K$, the approximation was found to be adequate. In the toad n^2 gave a better fit (Frankenhaeuser, 1962).

Figure 1, taken from Hille (1971), shows the voltage dependence of $\underline{m}$, $\underline{h}$, and $\underline{n}$ and the corresponding time

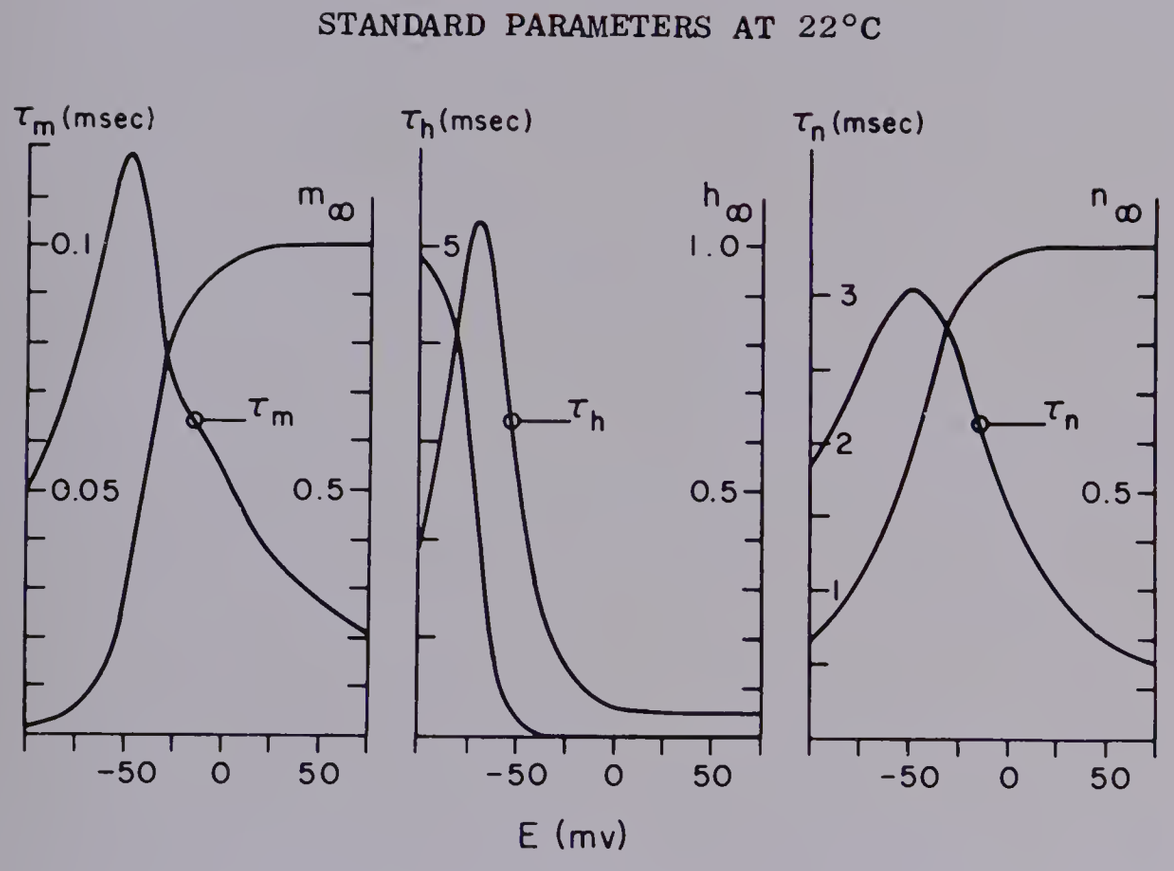


Figure 1. Voltage and time dependence of m, h, and n. Note, in this thesis that h_∞, m_∞, and n_∞ correspond to h, m, and n. Figure taken from Hille (1971).

constants. It should be noted that these are empirical constants and, as such, vary somewhat from node to node. The reader should become familiar with these curves because they will be referred to throughout this thesis.

Equations 8,9, and 24 may be brought together to give the total ionic current as:

$$i_i = \bar{P}_{Na}\, m^3 h\, [Na]e\, (F^2E/RT)\, (\exp[(E-E_{Na})\, F/RT]-1/\exp[EF/RT]-1)$$

$$+ \bar{g}_K n^4 (E-E_K) + \bar{g}_L (E-E_L) \qquad (25)$$

The upper part of Figure 2 shows the total ionic currents minus the leakage current, which are produced while in the voltage clamp mode, by stepping the membrane voltage to different values. Using procedures described in Chapter 3, the total ionic current may be mathematically separated into the potassium and sodium current components which are shown in the lower part of Figure 2.

Although the ability to separate the sodium and potassium currents mathematically with results similar to those obtained experimentally lends support to the model, the best test of the model is how sucessfully it can

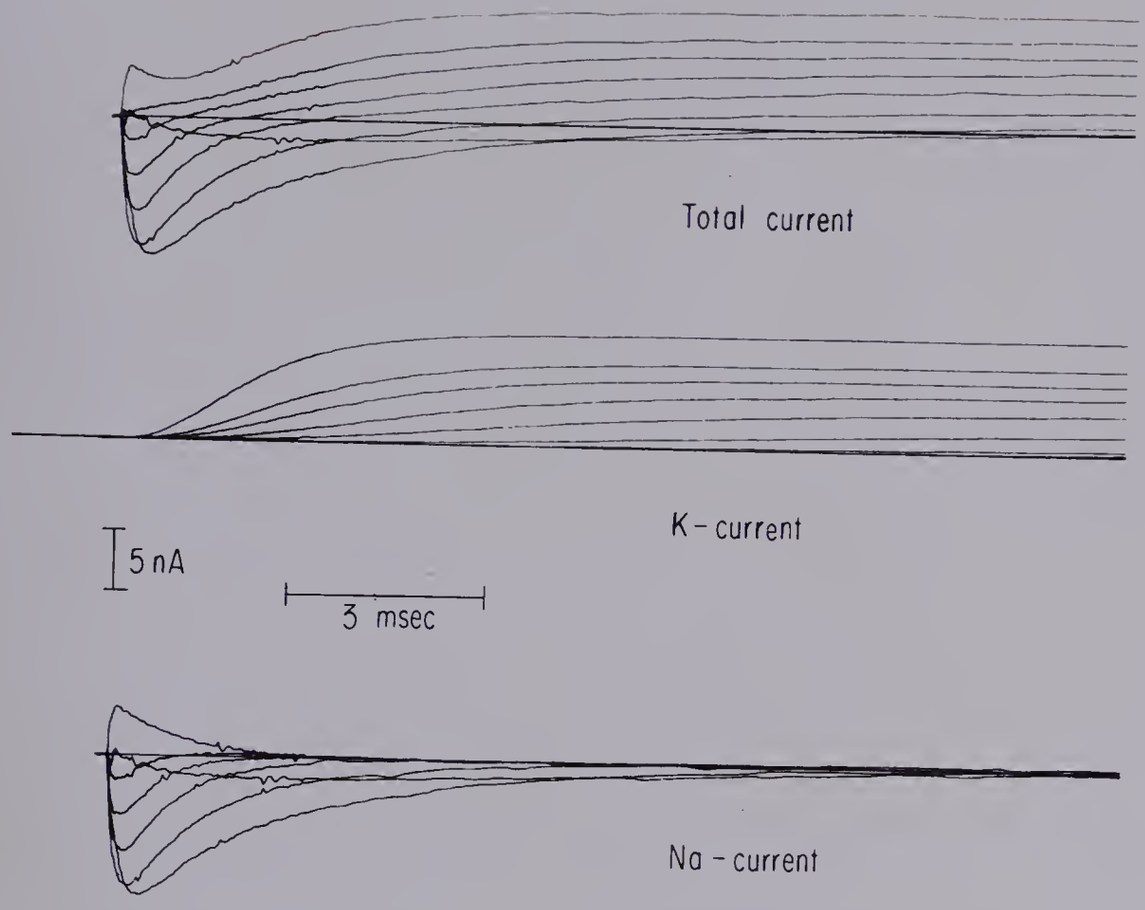


Figure 2. Voltage clamp currents. Upper family of curves produced a applying depolarizing steps from -50 to +69 mV in amplitude. In this figure, currents above the baseline are outward and positive, while those below the baseline are inward and negative. The potassium currents (middle curves) were derived mathematically from the model and subtracted from the total currents to leave the sodium currents. The leakage current has been removed from all records. T=6°C. Axon 82

reproduce a normal action potential. Using numerical integration methods with the appropriate boundary conditions, equation (2) giving the total membrane current flow may be solved with the ionic current being given by equation (25). The results of such an integration along with a recorded space clamped action potential taken from Dodge (1963) are shown in Figure 3. Although there are some differences between the two action potentials the mathematical model appears quite sufficient to describe the nodal action potential.

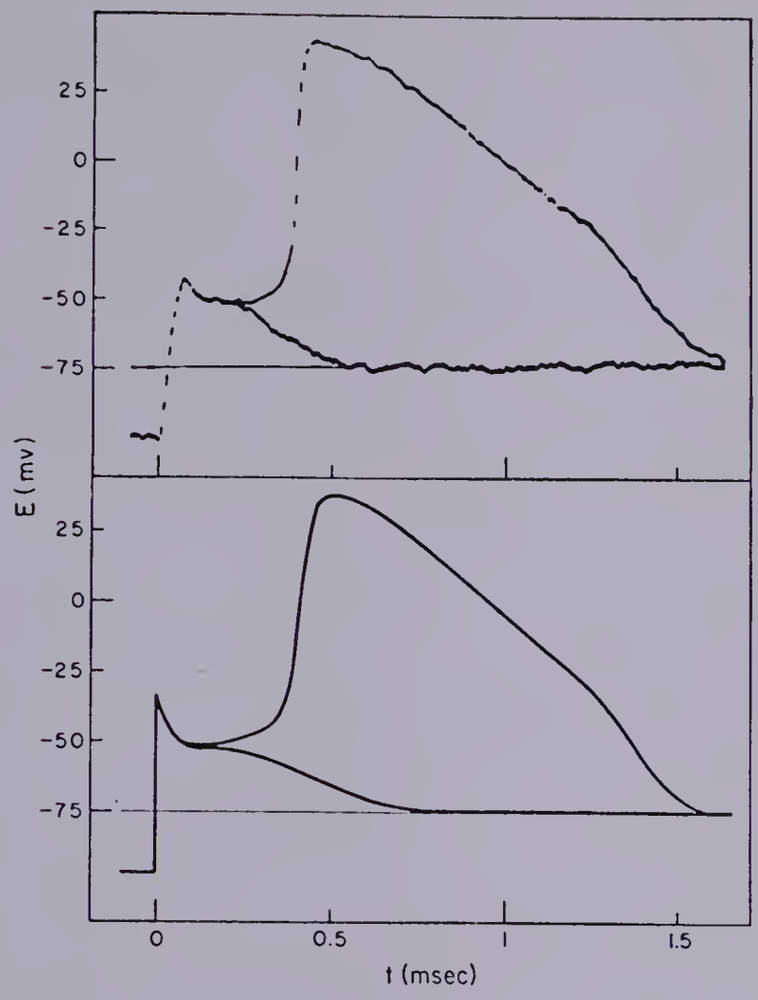


Figure 3. Subthreshold and suprathreshold nodal responses. Upper curves show the nodal action potential and subthreshcld response obtained experimentally. Lower curves are the same responses calculated from the model. Responses initiated from -105 mV. Figure from Dodge (1963).

Chapter 2

DRUG STABILIZATION OF EXCITABLE MEMBRANES

There have been many theories formulated to explain how narcotics (the term narcotic is used here to denote a cellular depressant and not only an opiate derivative) depress cellular activity, but most of these have suffered from an inability to specify how electical activity in electrically excitable cells is blocked. Perhaps the best known of the theories of narcosis is the Meyer-Overton lipoid solubility theory (Butler, 1950; Henderson, 1930; Hober, 1945; Meyer and Gottlieb, 1926; Seeman, 1966, 1972). Proponents of this theory maintain that narcosis occurs whenever a narcotic reaches a certain molar concentration constant in the membrane lipids. This constant is dependent on the type of cell studied, but is independent of the type of drug used providing a sufficient concentration can be reached in the membrane. But, as has been pointed out (Butler, 1950), this is not really a theory but a 'rule' as it is not stated how narcosis is achieved. Most of the "physical" theories of narcosis (Butler, 1950) suffer similar difficulties because their proponents have concentrated more on correlating physical properties of narcotics with some measure of narcosis, than in finding how these agents produce narcosis. "That is they are descriptive rather

than informative" (Charnock, 1973). I should now like to discuss in some detail one theory which attempts to deal with how narccsis may occur.

The Permeability Theory of Narcosis

In 1907 Hober found that drugs such as chloroform, phenyl carbamate, and ethyl carbamate could oppose the production of injury currents in muscle by potassium salts, thus demonstrating that these drugs could reduce membrane permeability to ions (cited by Shanes, 1958 a). Lillie (1923) later suggested that "stabilization" of the membranes was the "essential physical condition underlying narcosis". The term "stabilization" was used to indicate that the membrane was somehow less susceptible to structural modification, and therefore the normal permeability increase which is responsible for the action potential (and for a number of other biological phenomena) could not occur as it required a change in membrane structure. An electrically excitable cell would therefore fail to propagate a response. Lillie did not suggest how the membrane was stabilized, but this is not surprising considering that at the time almost nothing was known of the membrane permeability or structure.

Important support for the permeability theory came from the work of Cole and Curtis (1939). Cole and Curtis

measured the transverse impedance of the squid axon during the action potential and found that the membrane resistance fell during activity. This finding gave substantial support to Bernstein's membrane hypothesis that an increase in membrane permeability was responsible for the action potential (Cole, 1968). Therefore, a reduction in permeability would certainly block the action potential.

Many studies had shown the ability of narcotics to block action potentials (for references see Butler, 1950) but it was not until after the development of the intracellular glass microelectrode (Ling and Gerard, 1949), which enabled precise measurements of the resting and action potentials in muscle and nerve, and the Hodgkin and Huxley (1952, d) model of excitability was formulated that more exacting tests of the permeability theory became possible. To this end, Thesleff (1956) investigated the action of several general anesthetics on thresholds and action potential production in frog skeletal muscle fibres. With drug treatment the threshold for excitation increased while the rate of rise of the action potential and the height of the action potential were reduced. Assuming that the Hodgkin and Huxley model was appropriate for skeletal muscle, Thesleff concluded that the anesthetics suppressed the normal increase in the sodium permeability mechanism which is responsible for the rising

phase of the action potential. Because of the parallel between the dose necessary to anesthetize frogs and the dose necessary to block the action potential, Thesleff further suggested that the mechanism of general anesthesia was a block of the sodium conductance increase in neurons within the central nervous system.

In 1958 Shanes (1958 a, b) extensively reviewed the actions of what he called "labilizers and stabilizers" on excitable cells. He classified drugs such as veratradine as labilizers because they tended to accentuate electrical changes. Reduction of external calcium also accentuates these changes and was classified as a labilizing procedure. Drugs such as local anesthetics, antihistamines, and tetraethylammonium, which reduce or slow electrical changes, were classed as stabilizers. High external calcium also has a stabilizing effect. Among the conclusions reached was that labilizers acted by increasing membrane permeability while stabilizers decrease this parameter. Drugs which block the action potential, other than those which depolarize, would therefore do so by decreasing the normal increase in sodium permeability responsible for the rising phase of the action potential.

Shanes et al (1959) applied the voltage clamp technique to analyzing the action of local anesthetics on

squid giant axons. Both procaine and cocaine reduced the early peak currents and the late steady state currents while the resting potential remained unchanged. Taylor (1959) also examined the action of procaine on voltage-clamped squid axon and presented results similar to those of Shanes et al. In neither study were the currents separated into the sodium and potassium components, nor was a kinetic analysis carried out. Taylor did show however that the time to peak of the early current was prolonged as was the time to 75% of the final steady state current values. The other parameter measured was the resting sodium inactivation which did not change with procaine treatment. Both Shanes and Taylor concluded that the major effect of the drugs studied was to reduce the maximum sodium and potassium conductances. The reduction of the sodium conductance is responsible for the block of the action potential but, as Taylor pointed out, the effectiveness of procaine is reduced because a decrease in the potassium conductance tends to increase excitability.

Since these initial pharmacological studies using voltage clamp methods were conducted, several other classes of narcotics have been subjected to a similar analysis on different nerve and muscle preparations and have yielded similar results (see Chapter five for further discussion and references). It thus appears that Lillie's initial hypothesis which was postulated in 1923 has been

proven correct. That is, the blockade of excitibility by narcotics is due to a reduction in the normal increase in permeability which is responsible for the upstroke of the action potential. This theory has proved very useful in accounting for the mechanism of both local and general anesthesia, and has lead to the conclusion of some investigators that local and general anesthetics have a common mode of action (Frank and Sanders, 1963; Frank, 1972).

Although a reduction in membrane permeability may account for the ability of narcotics to block excitability, the mechanism by which the permeability is affected remains unknown. Recent experiments using internal perfusion of squid giant axons have shown that the axoplasm is not essential for either action potential production or block by drugs (Oikawa et al, 1961; Narahashi et al, 1967; Gruener and Narahashi, 1972; Narahashi and Frazier, 1971; Tasaki et al, 1962). In experiments in which most of the axoplasm was removed and the axon perfused with artificial solutions, such drugs as the local anesthetics, chlorpromazine, pentobarbital, and tetrodotoxin (TTX) are just as effective in blocking the action potential, when they are applied externally, as they are when the axoplasm is present. Although these experiments demonstrate that narcotics act directly on the membrane, they do not preclude the possibility that some

narcotics may also influence permeability by influencing metabolic processes within nerves (Krnjevic, 1972). TTX, however, acts only on the membrane as internal perfusion studies have shown that TTX is unable to cross the membrane (Narahashi et al, 1967).

There are some drugs such as the antiepileptic agents which, judging by the action potential, appear to stabilize excitable membranes without reducing membrane permeability. In the squid giant axon diphenylhydantoin (.6mg/ml) has no effect except to produce a slight increase in excitability. However, when the concentrations of calcium and magnesium are reduced and the axon is spontaneously active, this same concentration of diphenylhydantoin will abolish the spontaneous activity (Korey, 1951). In frog nerves, repetitive stimulation can lead to a condition whereby a single stimulus can evoke repetitive firing. In nerves pretreated with diphenylhydantoin this repetitive activity is prevented (Toman, 1952).

In trying to quantitate the action of antiepileptic drugs on spontaneously active squid axon, Rosenberg and Bartels (1967) determined a "ratio of effectiveness" which was given as the ratio of the minimum drug concentration necessary to block the action potential, to the drug concentration necessary to block spontaneous activity

produced by seawater containing low calcium and magnesium. This ratio was over 100 for diphenylhydantoin, twenty for trimethodione, thirty-three for phenobarbital, and five for barbital. This certainly suggests that a mechanism distinct from a simple reduction in the sodium conductivity is operating with these agents. Although a reduction in the normal increase in sodium conductivity can reduce spontaneous activity, other suggestions have included stimulation of the sodium pump or a modification of the sodium system kinetics (Shanes, 1958b; Woodbury, 1969). Unfortunately, there is insufficient information to decide among these alternatives.

Because calcium seems to play such an important role in membrane excitability much attention has been focused on the interaction between drugs acting on membranes and calcium (Shanes, 1958a, b; Seeman, 1972). Calcium is required for the proper functioning of many membrane processes including excitation (Manney, 1967; Frankenhaeuser and Hodgkin, 1957; Frankenhaeuser, 1957; Brink, 1954). Since a lowering of the external calcium concentration increases excitability whereas increasing the concentration of this ion has the converse effect, it has been suggested that calcium may either regulate membrane permeability and thus excitability or that it is intimately involved in the regulatory process (Blaustein and Goldman, 1966b; Brink, 1954; Goldman, 1964; Tobias,

1964).

Feinstein (1964) suggested that local anesthetics may act by competing with calcium for critical binding sites on phospholipids. He found that both phosphotidyl serine and cephalin could bind calcium by measuring the amount of calcium moved from an aqueous phase to a chloroform phase. This translocation of calcium was prevented by the addition of a local anesthetic such as procaine. Since both local anesthestics and calcium were found to bind to the polar groups on the phospholipids, when both agents are present they compete for these sites. Feinstein postulated that on biological membranes calcium and local anesthetics may compete for spike generating sites, but calcium alone will support regeneration; the local anesthetics produce a block.

Blaustein and Goldman (1966a), following Feinstein's work, demonstrated a competitive action between procaine and calcium on lobster axons. Both calcium and procaine increase threshold and shift the sodium conductance versus membrane potential curves in the direction of depolarization. However, elevated calcium concentrations do not usually reduce the maximum sodium conductance whereas procaine does. This reduction in sodium conductance is an inverse function of the calcium concentration. That is, the greater the calcium

concentration the smaller the reduction in scdium conductance produced by prccaine. The potassium system showed similar effects.

Statement of the Problem

The objective of this thesis was to carry out a voltage clamp analysis of the stabilizing action of different classes of narcotics and antiepileptic drugs on frog myelinated nerves in order to clarify by what mechanism of action these drugs exert their effects. Part of this objective was to test the hypothesis that calcium or the sites that bind calcium may play an important role in the membrane action of some of the drugs tested.

Chapter 3

METHODS AND MATERIALS

Nerve Fibre Preparation

A section of sciatic nerve 3-4 cm. long was removed from the upper leg of the frog *Rana pipiens* and placed on the glass dissecting plate of a binocular microscope. Under dark field illumination the nerve bundle was desheathed and a single fibre isolated by the method of Stampfli (1969). At least one internode and the greater part of the two surrounding internodes were isolated. The remaining fibres were trimmed back leaving the single fibre between two nerve bundle stumps. The single fibre was then transferred under Ringer solution to the recording chamber shown in Figure 4. The node under investigation was mounted over compartment A and held in place with a small dab of Vaseline on the stumps in compartments C and E. Vaseline seals were then formed on all ridges using a 30 gauge hypodermic needle. The recording chamber was then mounted in a brass enclosure which served to shield the chamber electrically and to maintain the temperature (see below). Electrodes were connected to compartments C, A, B, and E with 3 molar KCl salt bridges in 1% agar. The level of the Ringer solution in the recording chamber was lowered until the Vaseline

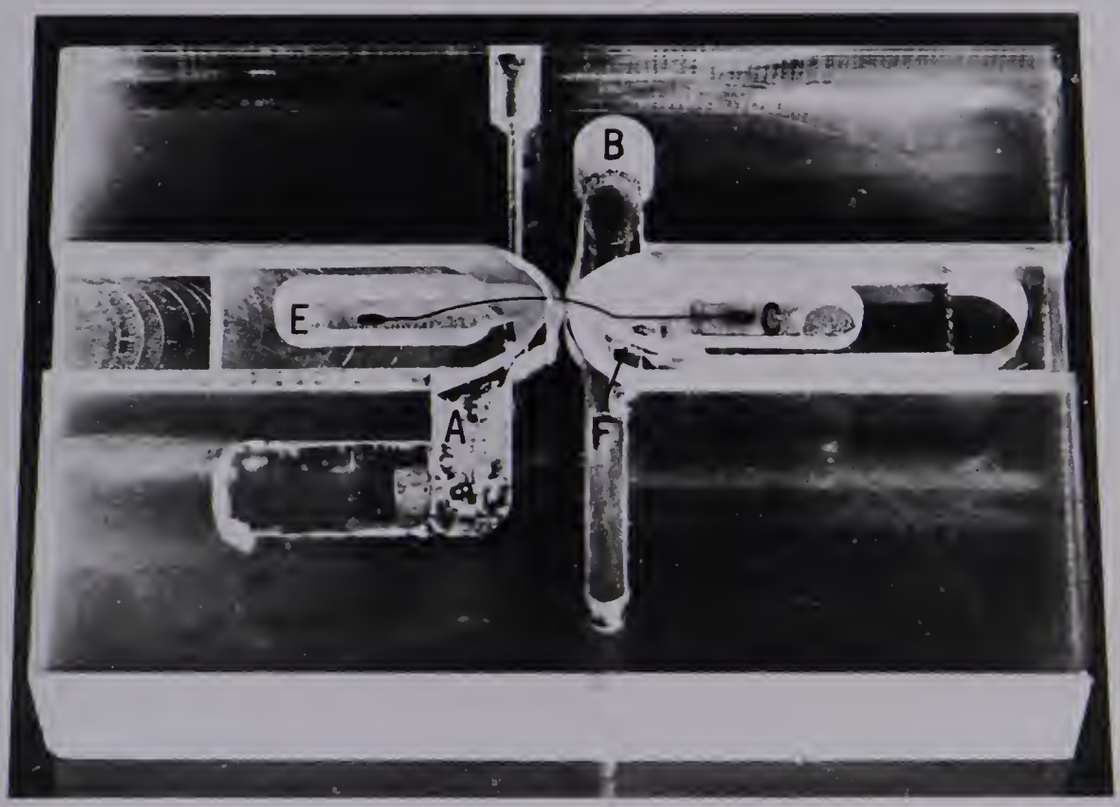


Figure 4. Recording chamber. The nerve is indicated by the dark line and the node by the break in the line over compartment A. Solutions were introduced through the hole which is shcwn in the upper part of the figure and are drawn off by a vacuum line mounted above A (not shown).

seals emerged, leaving five isolated compartments. The fluid remaining in compartment F was removed by suction leaving an air gap. In order to prevent resistive changes in the nodes in compartments E and C a 1% solution of formalin in isotonic KCl was added to these compartments for 30 seconds after which isotonic KCl alone was added (Hille, 1967a).

The recording chamber was modelled after that of Nonner (1969). It was constructed from plexiglas (Rohm and Haas) and designed such that the width of the compartments could be varied. The compartments widths were usually adjusted to 200, 250, and 200 micrometres for compartments A, B, and F respectively. The thickness of the ridges on which the nerve rested were 90 micrometres. A light coating of Vaseline applied to the chamber parts before assembly insurred that the they remained in place after adjustment and that the compartments did not leak.

Drugs

The following drugs were used for the research reported in this thesis:

sodium bromide — Fisher Scientific

chlorpromazine hydrochloride — Poulenc Ltd.

chloral hydrate — Winnipeg General Hospital

diphenhydramine hydrochloride — Sigma

sodium diphenylhydantoin	Park Davis
sodium phenobarbital	BDH
sodium pentobarbital	BDH
procaine hydrcchloride	Matheson
strychnine sulphate	BDH
trichloroethanol	Aldrich

Solutions

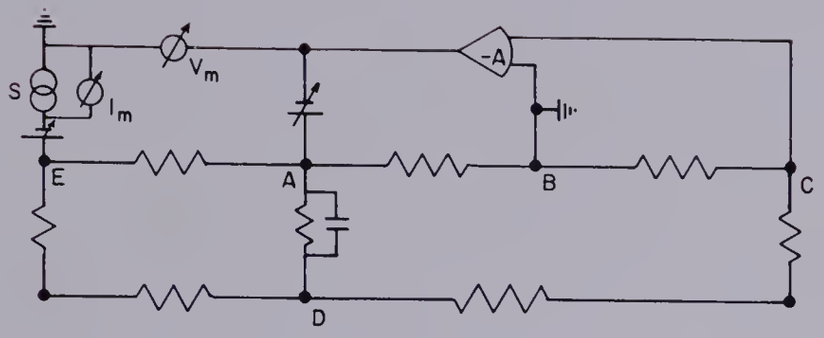
The Ringer solution used had the following composition in millimoles: NaCl, 111.8; KCl, 2.5; $CaCl_2$, 1.8; glucose, 11.1; tris (hydroxymethyl) aminomethane free base, 1. The pH of all solutions was adjusted between 7.1 and 7.2 at 20° C. by addition of hydrochloric acid. Drug solutions were made up by dissolving the drug to be used in Ringer solution (while it was being made) and then adjusting the pH to the range used in this study. Drug solutions made up in this way were therefore hypertonic to normal Ringer solution. Diphenylhydantoin was made up by dissolving the drug in the solvent supplied by the manufactuer (propylene glycol and sodium hydroxide at pH 11.5) and then adding the required amount of this solution to the Ringers solution. Solutions containing higher or lower concentrations cf calcium were made by varying the amounts of $CaCl_2$ added. The water used to make up solutions was obtained frcm the labratory distilled water supply and passed through two mixed bed resins before use.

The specific resistance of this water was between 9 and 18 megohms. The osmolarity of all solutions was checked using a freezing point depression osmometer and were found to lie between 220 and 250 milliosmoles.

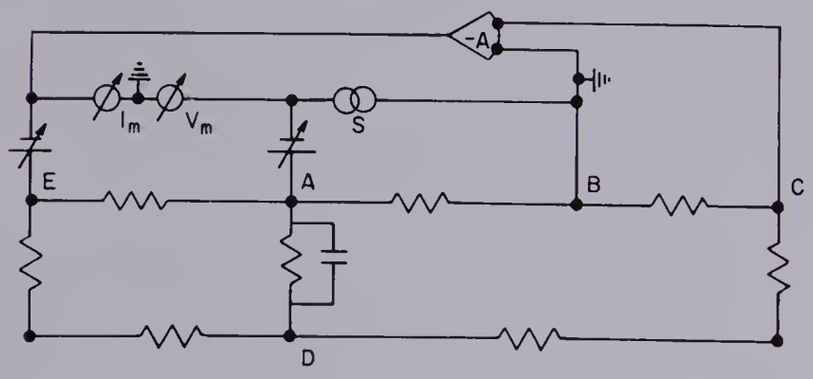
Electronic Control and Measurement System

The experiments reported in this thesis are of two types: current clamp experiments in which voltage is the dependent variable, and voltage clamp experiments where current is the dependent variable. To perform these experiments either the current or the voltage respectively must be controlled. Frankenhaeuser (1957) and later Dodge and Frankenhaeuser (1958) developed the methods necessary to carry out these experiments on single myelinated nerve fibres. The methods used in this thesis are based on those of Nonner (1969) which are similar in principle to those of Dodge and Frankenhauser. These methods are briefly described below.

Figure 5 is a schematic diagram of the electronic control circuits used. The letters A, B, C, and E correspond to the compartments of the recording chamber shown in Fig. 4. The letter D stands for a point in the axoplasm. The resistances Rea and Rab represent the resistance of the Vaseline seals between compartments. Rbc is the lumped resistance of the Vaseline seals between



Current clamp circuit



Voltage clamp circuit

Figure 5. Clamp circuits. Circuits used to control the voltage across the nodal membrane. Triangles indicate operational amplifiers. E,A,B, and C, correspond to the compartments in Figure 4.

B and C and the air gap formed by comparment F. The amplifier, marked A, had a voltage follower input with a gate current less than one picoamp and an input impedance of greater than 10^{11} ohms. The maximum gain was one thousand with a bandwidth of DC to 10^5 Hertz .

In the current clamp circuit the amplifier is connected in a negative feedback configuration with point C as the summing junction. By the action of negative feedback, the amplifier injects a voltage at point A to keep pcint C and therfore point D at ground potential. If the node in compartment C is bathed in isotonic KCl, then the potential measured at point A with respect to ground is equal to the inverted resting membrane potential. All potentials in this thesis are on the E scale where E is given as inside potential minus outside potential.

If a constant voltage is now applied at point E by the stimulator, a constant current given by Ved/Red is injected into the membrane at point A. In this manner the node may be polarized. If a depolarizing pulse of suffient magnitude is applied to the node then an inverted action potential of normal amplitude will appear at point A. This may be easily measured by an oscilloscope connected to the output of the amplifier.

In the voltage clamp mode the constant voltage output

of the stimulator is applied to point A and the output of the amplifier applied tc point E. When a pulse is injected at A the amplifier injects a current at E given by Ved/Red in order tc maintain point C and therefore point D at ground potential. Since the voltage measured at point E is proportional to the current, the current may be calculated by assuming that the nodal resistance is equal to 40 megohms (Tasaki, 1955).

The batteries shown in Figure 5 are used to balance the circuit such that when it is switched back and forth from the current clamp to the voltage clamp the circuit remains in balance. In the current clamp mode when compartment C contains isotonic KCl the potential at A is adjusted to 75 millivolts. This value is close to the normal resting potential cf these fibres (Dodge, 1963). The battery at A is then used to adjust this potential to zero. The circuit is now switched to the voltage clamp mode and the battery at E adjusted so the output of the amplifier still reads zerc. The circuit at this point is now fully balanced and cnly needs slight adjustment when the amplifier drifts.

Recording

In order to facilitate analysis of the experiments, all data was collected and stored on a PDP-8E computer using methods very similar to those described by Hille (1967a). The methods used will be briefly described in this section.

In order to process and store the signals from the experiment they must be converted from analogue to digital signals. This is accomplished in the experiments by leading the voltage and current outputs to the input of a multiplexed 10 bit analogue to digital converter (A-D converter) in the computer which can sample from the incoming signals at a predetermined rate, thus converting the analogue value to a digital 'point' which is stored in memory.

The signals to be processed from a typical voltage clamp run are shown in Figure 6. The voltage signal is taken from the output of the stimulator and consists of a 40 millisecond hyperpolarizing prepulse used to remove the resting sodium inactivation of the sodium system and a 30 millisecond test pulse. Since the duration of these pulses was known, the only other information required was the amplitude of each. This was obtained by sampling the voltage pulses and averaging these values for each voltage

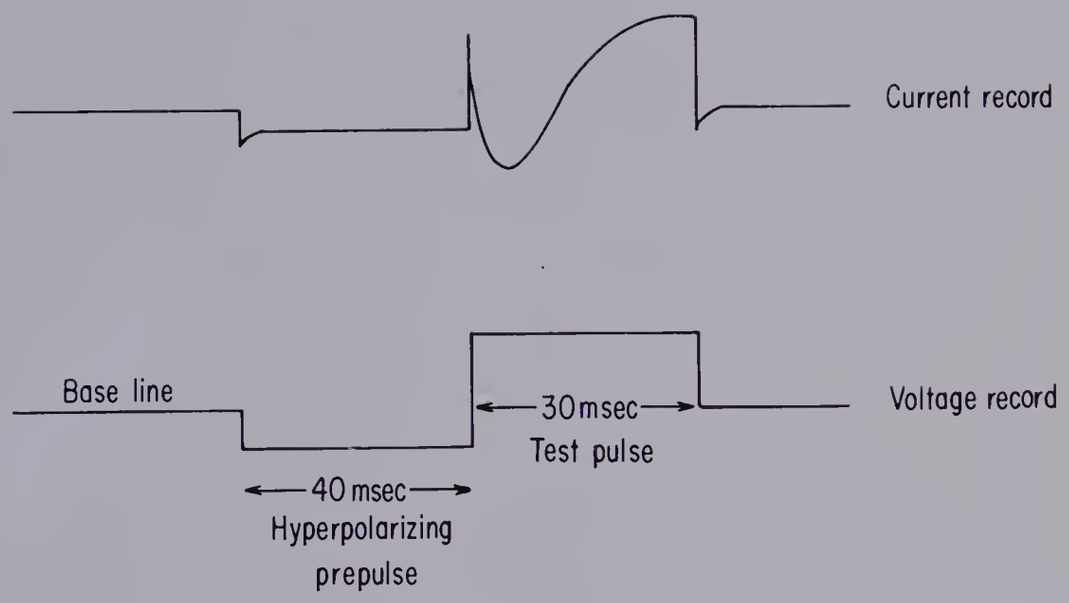


Figure 6. Voltage clamp signals. This is a schematic of the voltage pulses applied to the node and the resulting currents to be recorded. Not drawn to scale.

step. The amplitude of each voltage step was thereby reduced to one 'pcint' which was saved.

The current signal from the output of the control amplifier during the prepulse was treated in the same manner as the voltage pulses. The current during the test pulse is different from the other signals in that it varies with time. Because of the high rate of change of the current signal during the early part of the test pulse a sample was taken every 30 microseconds and stored. After 100 points had been taken the sampling rate was changed to one pcint every 300 micrcseconds.

After the sampling was complete and the mean values computed, the data was transferred tc magnetic tape for storage. The amplitude of the test pulse was then changed and the next run started. A voltage clamp series consisted of 20 runs with the amplitude of the test pulse being varied from -75 millivolts (the holding potential) to +77 millivolts, in 8 millivolt steps. At the end of a series the solution surrounding the node was changed and after a suitable time (usually 3 minutes) a new series was carried out.

(A listing of the programs used for the collection, storage, and analysis of data in this thesis is available on request frcm the Department of Pharmacology, University

of Alberta.)

Temperature

Because, at room temperature and at the sampling rate used, the rate at which the sodium system turns on is too high to be accurately recorded, the temperature of the node was kept between 3 and 6 degrees C. The temperature was controlled by fluid, from a thermostatically controlled bath which was circulated through the brass enclosure surrounding the recording chamber. To ensure that the temperature of incoming test solutions was the same as that of the node under observation, the solutions were added via a 2 cc. syringe mounted in a heat exchanger cooled by the same fluid circulating in the brass enclosure.

It should be pointed out that reducing the temperature of the nodal membrane does not appear to produce any effects other than those expected from the Q_{10} values of the conductance and kinetic parameters (Cole, 1968).

Data Analysis

Analysis of the data was usually carried out by graphical means in a sequence of several steps. The procedures used are similar to those of Dodge (1963) and Hille (1967a). The first step was the removal of baseline offsets, the capacitive transient, and the leakage current. Baseline offsets, due to imbalances in the measuring and recording network, were removed by sampling the baseline voltage and current signals after each clamp pulse and storing the mean values. These mean offsets were later subtracted from the current and voltage signals. The capacitive transients were removed by taking the transient obtained when the test pulse was equal to the membrane potential and scaling this to the magnitude of the test pulse. Because noise is also scaled up with this procedure the transient was first digitally filtered.

The leakage conductance was obtained from the amplitude of the current and voltage signals during the prepulse. The leakage conductance was assumed to be constant so the leakage current was given by the product of the conductance and the test voltage. This current was then calculated for each test pulse and subtracted from each current 'point' during that pulse.

The second step was to plot the current records in

real time on an X-Y recorder. From these plots the early peak current (I_{Na}), the late steady state currents (I_K) and the sodium equilibrium potential (E_{Na}) were obtained. The peak and steady state current values were then used to determine the sodium and potassium 'conductances' by evaluating the the following expression,

$$Px[X]e\ F^2/RT = Ix/E(\exp[(E-Ex)F/RT] - 1/\exp[EF/RT] - 1) \quad (26)$$

where X stands for either sodium or potassium. In my experiments then, $\bar{g}_K$ and $\bar{g}_{Na}$ stand for $Px[X]e\ F^2/RT$.

The third step was to perform a log-log plot of current versus time. From this plot the value of the potassium time constant (T_n) may be obtained. Dodge (1963) had found that if the membrane was hyperpolarized for a sufficient length of time such that the steady state value of n approached zero then the potassium current (I_K) could be described by:

$$I_K = A(1-e[-t/T_n])^4 \quad (27)$$

where A is the amplitude factor and T_n is the time constant for the turning on of the potassium system. Therefore if the total current minus the factors in step one is plotted logarithmically then the curves for I_K will

be identical except for differences in T_n and A. A template of equation 26 can be made and slid over the log-log plot to determine A and T_n. In some cases I_K did not level off as would be expected from equation (27) but continued to rise. In these cases the template was fitted only to the rising portion of the curve.

The next step was the mathematical separation of the sodium currents from the potassium currents. This was accomplished by using the value of T_n and A obtained from the log-log plot and then calculating the value of I_K at each 'point' and subtracting this from the current record. The remaining current, according to the model presented in Chapter 1 is I_{Na}. A plot of this current versus time confirms that the separation was carried out correctly. That is, the currents are similar to those predicted by the model. The currents are also similar to those produced under the influence of tetraethylammonium which specifically reduces $\bar{g}_K$ (Hille, 1967a, b). The last step was to plot the sodium currents in a semilog form to extract the sodium time constants T_h and T_m. From the final slope of the semilog plot the time constant T_h is obtained. A line parallel to this slope but log (.5) below it intersects the rising phase of the sodium current at a point where $t/T_m = 1.58$ and T_m is therefore determined by this procedure (Hille, 1967a).

Errors

The errors in both measurement and analysis have been dealt with by Dodge (1963), Hille (1967a), and Nonner (1969) and will only be briefly described below.

The errors may be divided into instrumental and analytical error. The major instrumental problem was drift in the input amplifier. To compensate for drift the sodium equilibrium potential was used as a reference point. In the frog node the sodium equilibrium potential is quite stable because the large internodal volume of the axoplasm prevents the build up of sodium in the nodal region.

The other instrumentation problem was the 'attentuation artifact' (Dodge and Frankenhaeuser, 1958). This artifact is produced when compartment B is too narrow, and has the effect of reducing the voltage across the node. If the width of compartment B is increased the feedback amplifier is unable to clamp the high frequency response of the node and the clamp becomes unstable. This artifact may be measured by replacing part of the sodium in Ringers solution with a nonpermeant cation such as tetramethylammonium and comparing the theoretical change in the equilibrium potential with the observed equilibrium potential. Alternatively, the height of the observed

action potential may be compared to the expected value of 115 mV. (Noonner, 1969). In my experiments the artifact varied between 10 and 30% and was adjusted accordingly.

The analytical errors arose in regard to the accuracy and reproducibility of the measurements made from the various plots. T_n was measured with an accuracy of 2.5% and when measured the reproducibility was between 3 and 6% depending on the amplitude of the potassium current and noise. As T_h is dependent on how well T_n is choosen, the accuracy of T_h is probably within 10%. T_m is probably accurate to within 5 to 10% as digital timing was used to start the A-D clock and the test pulse at the same time.

Chapter 4

RESULTS

Barbiturates

The barbiturates are an interesting group of drugs because from within this class can be found convulsants, antiepileptic agents and general depressants (Ariens, 1964; Goodman and Gilman, 1970). Although there is an obvious diversity of action produced by only slight structural modifications, only two of the barbiturates have been examined using voltage clamp techniques. Blaustein (1968a) found that in lobster axons, both sodium thiopental and sodium pentobarbital reduced the maximum sodium and potassium conductances and prolonged the time to initial peak current (this time gives a rough estimate of T_m). Similar results with sodium pentobarbital have been reported for squid axons (Narahashi et al , 1969).

In my experiments I examined the action of sodium phenobarbital and sodium pentobarbital. Although both of these drugs are general depressants and are used clinically for this effect, phenobarbital has the additional property of being useful as an antiepileptic agent at doses slightly below those necessary for sedation.

The current-voltage relations for the peak and steady state currents before, during and after .5 mM phenobarbital are shown in Figure 7. The major effects, as can be clearly seen, are a reduction in both the peak and steady state currents with a poor recovery for the latter and an increase in the threshold for the flow of inward sodium current. In four other axons studied with phenobarbital, the steady state currents were not usually affected even with concentrations up to and including 1 mM. When reductions in the steady state currents were observed they were due to reductions in $\bar{g}_K$. In Figure 7, for example, $\bar{g}_K$ was reduced to 87% of control.

The reduction in the peak current and at least part of the shift in the current-voltage relation with phenobarbital is due to a reduction in $\bar{g}_{Na}$. In .5 mM phenobarbital the reduction in $\bar{g}_{Na}$ varied from 75 to 85% of control values while in 1 mM phenobarbital it varied between 50 and 70% of control in different axons. Although $\bar{g}_{Na}$ was always reduced (5 experiments), and in some axons also $\bar{g}_K$ (2), $\bar{g}_L$ was never found to vary by more than plus or minus five percent of control.

It should be pointed out that the above reductions in $\bar{g}_{Na}$ are not sufficient to block nodal action potentials. Computer calculations carried out using the data of

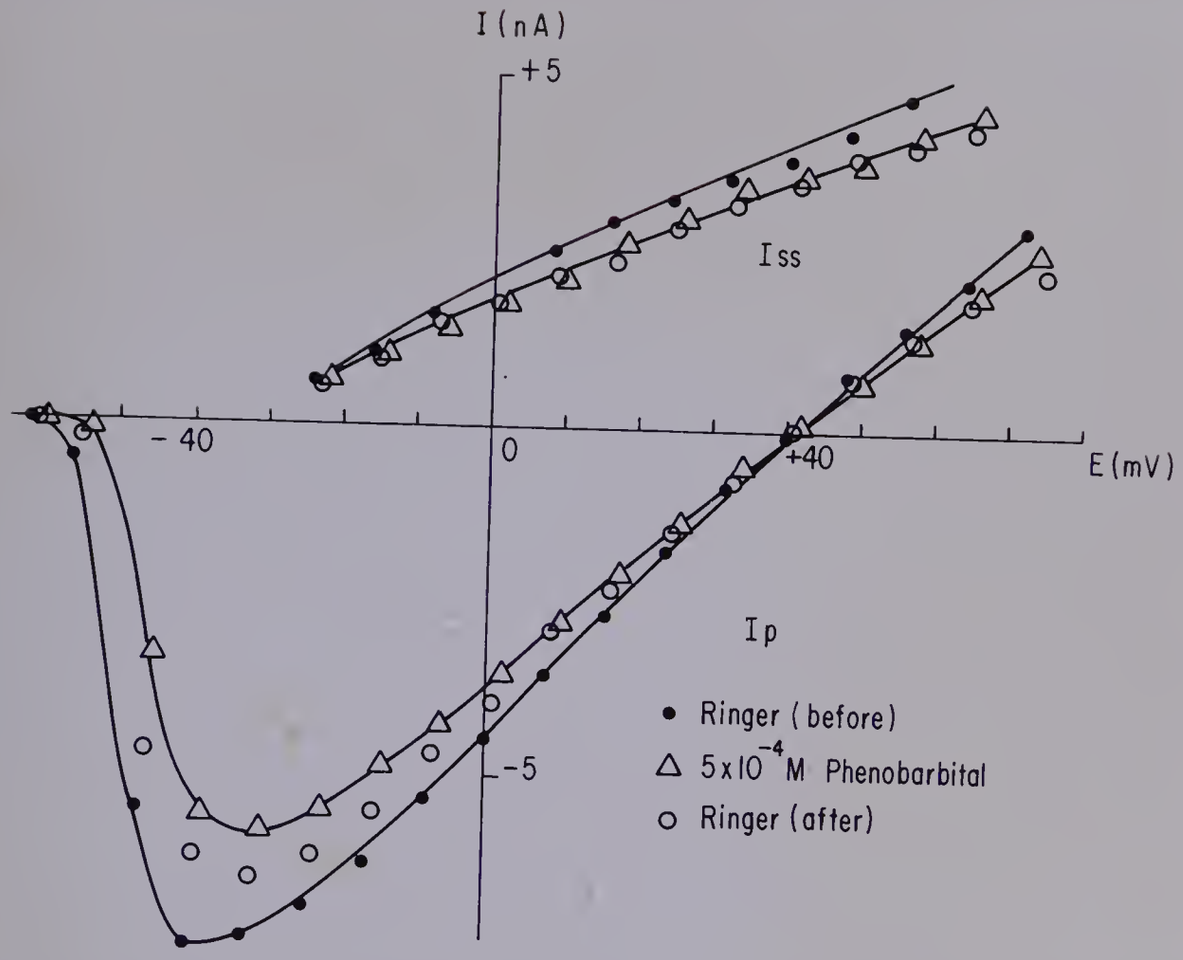


Figure 7. Current-voltage relations in phenobarbital. Peak (Ip) and steady state (Iss) currents before, during and after .5 mM phenobarbital. T=5°C. Axon 67

Frankenhaeuser's for the toad node, (Frankenhaeuser and Huxley, 1964), have shown that a 50% reduction in $\bar{P}_{Na}$ only reduces the amplitude of the action potential by 8 mV. In my experiments I have kept the drug concentrations small because large reductions in the currents would make complete analysis impossible.

The effect of .5 mM phenobarbital on the sodium and potassium time constant curves is shown in Figure 8. These data are obtained from the same runs shown in Figure 7. In this analysis the only curve which is affected is that for T_m which is shifted along the voltage axis in the direction of depolarization. That is, the time necessary for the sodium current to turn on is increased. The shift of the T_m curve according to the model in Chapter 1 is indicative of a shift in the curve relating $\underline{m}$ to membrane potential in the direction of depolarization. It is this shift in $\underline{m}$ which would explain the increased threshold for inward sodium current. The membrane would have to undergo a greater depolarization to raise m to a value sufficient to detect any inward sodium current.

Other axons (4) yielded similar results except that in two axons T_h was shifted by 3 mV in the direction of depolarization while in another axon T_n was shifted by 10 mV in the same direction. In all axons studied T_m was shifted to the right.

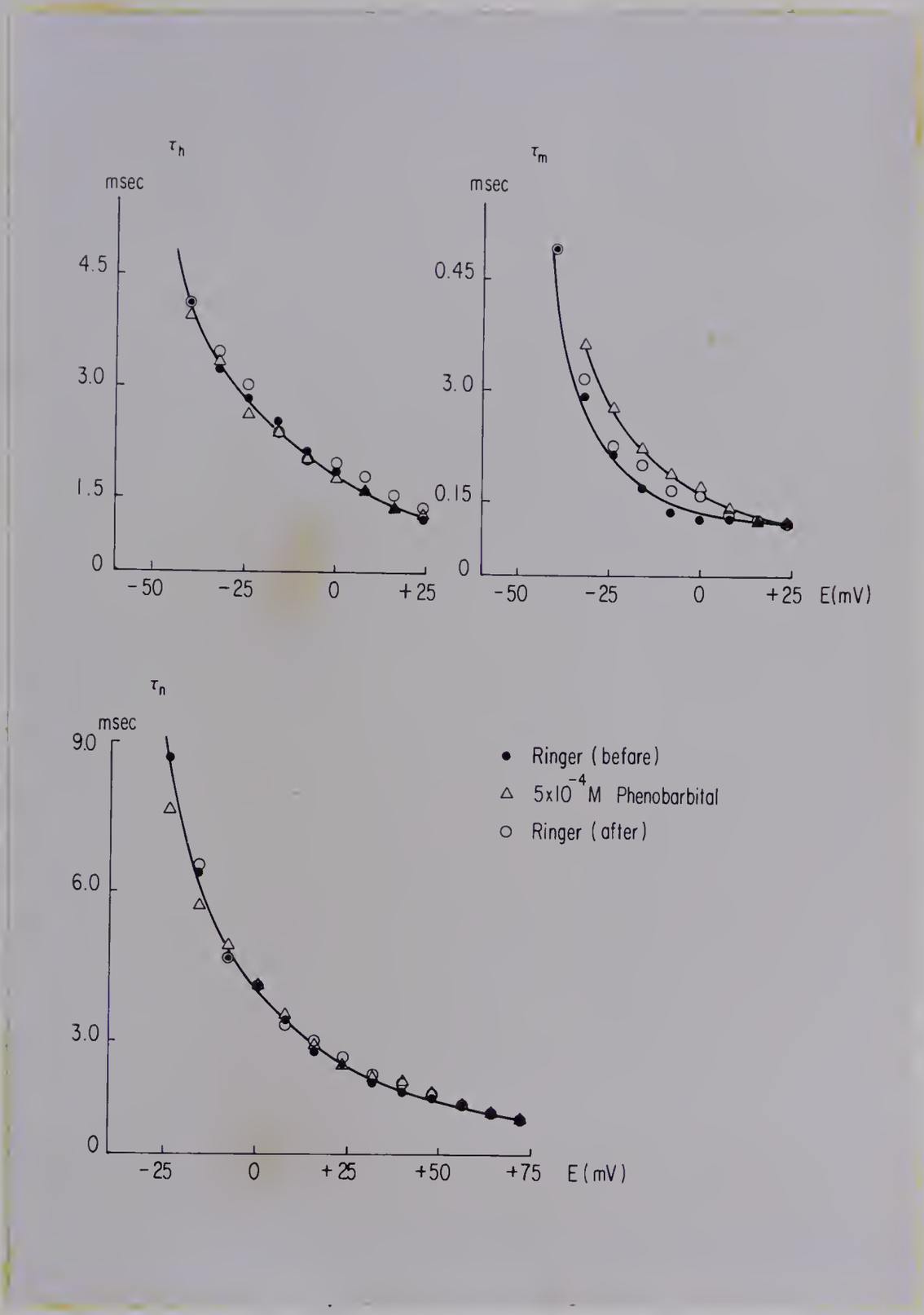


Figure 8. Sodium and potassium time constant curves in phenobarbital. Data from the same runs shown in previous figure.

In order to investigate the antiepileptic action of phenobarbital, nodes were bathed in phenobarbital Ringer made up with .36 mM calcium instead of the normal 1.8 mM calcium. In squid axons a reduction in the calcium concentration leads to spontaneous activity which has been used as a model for epileptic discharges. Frog nodes bathed in Ringer containing reduced calcium concentrations do not show spontaneous activity unless tetraethylammonium is also present to reduce the potassium currents (Bergman et al, 1968). Although spontaneous activity is not observed at the frog node, Ringer containing reduced concentrations of calcium still produces a shift in the sodium time constants in the direction of hyperpolarization as it does in the squid axon. The lower part of Figure 20 shows the typical shift in T_h and T_m seen in nodes exposed to Ringer containing .36 mM calcium. Hille (1967a, 1968b) investigated the effect of calcium on frog nodes and found an average shift of 20 mV in the sodium time constants per decade change in calcium concentration. In my experiments 9 mM calcium produced 8 to 12 mV shifts to the right while .36 mM calcium produced 6 to 10 mV shifts in the opposite direction. T_n was not affected by altering the calcium concentration. This finding is in agreement with Hille's (1967a, 1968b) results with Rana pipiens . Brisman and Frankenhaeuser (1972) on the other hand found that with Xenopus laevis the curve relating n to membrane potential was shifted by

changing the external calcium concentration. It appears therefore, that there is a species difference between Xenopus and Rana nerves in their response to calcium.

Figure 9 shows the peak and steady state current-voltage relaticns before, during and after treatment with .5 mM phenobarbital in .36 mM calcium Ringer. It should be noted that the contrcls before and after exposure to the drug contained 1.8 mM calcium. As can be seen there is little difference between this figure and Figure 7 except that the combinaticn of .36 mM calcium Ringer plus drug reduced the peak current to a greater extent, threshold for inward sodium current was not affected. As in normal Ringer both $\bar{g}_{Na}$ and $\bar{g}_{K}$ are reduced while $\bar{g}_{L}$ remained unaffected. In this example $\bar{g}_{Na}$ was reduced to 72% of control while $\bar{g}_{K}$ was reduced to 84% of control. Although .36 mM calcium by itself does not lower $\bar{g}_{Na}$, the combinaticn cf lcw calcium and .5mM reduced $\bar{g}_{Na}$ slightly more than did phencbarbital alcne in three out of five axons studied.

The time ccnstant curves for the sodium and potassium systems are shown in Figure 10. These data are obtained from the experimental runs shown in the previous figure. With 1.8 mM calcium Ringer as control there is no difference between the test sclution and the controls. However, when this same axon was bathed in .36 mM calcium

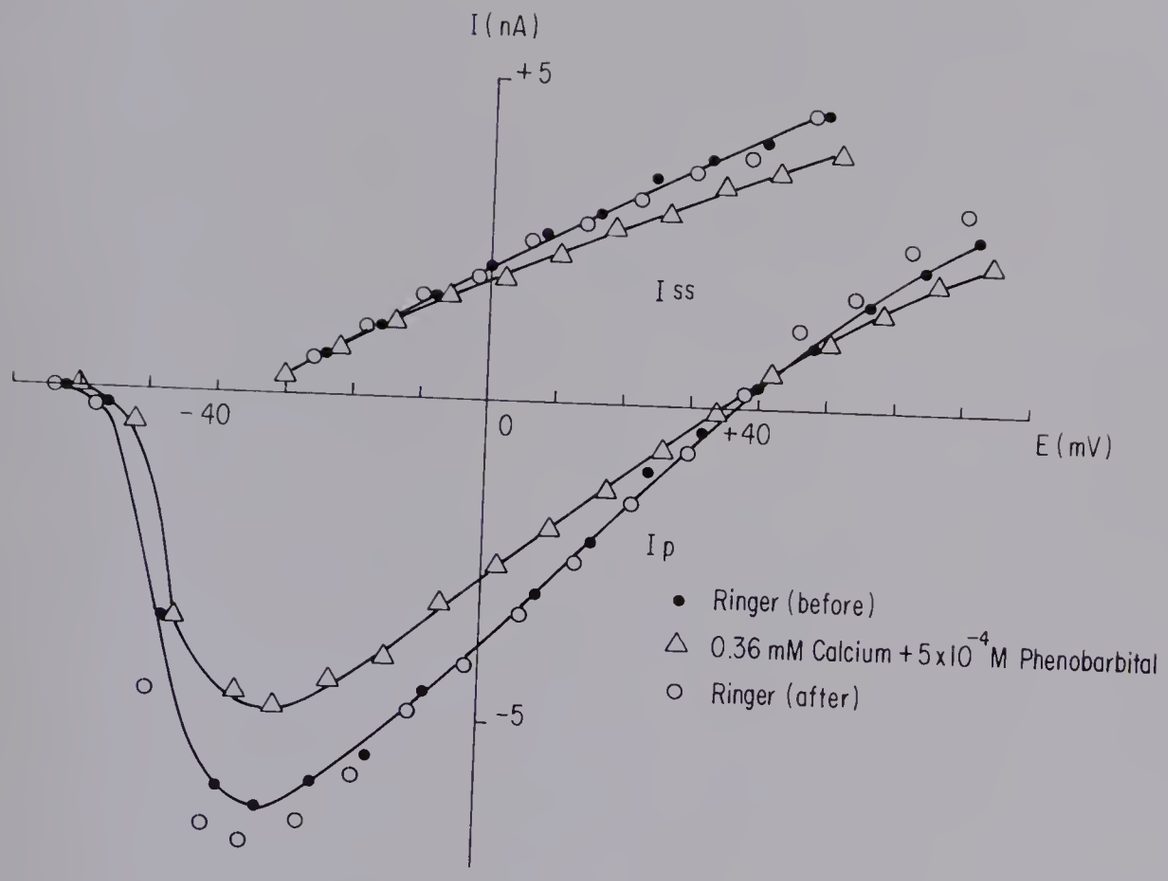


Figure 9. Current-voltage relations in low calcium phenobarbital Ringer. Peak (Ip) and steady state (Iss) currents in .36 mM calcium plus .5 mM phenobarbital Ringer. T=5°C. Axon 67

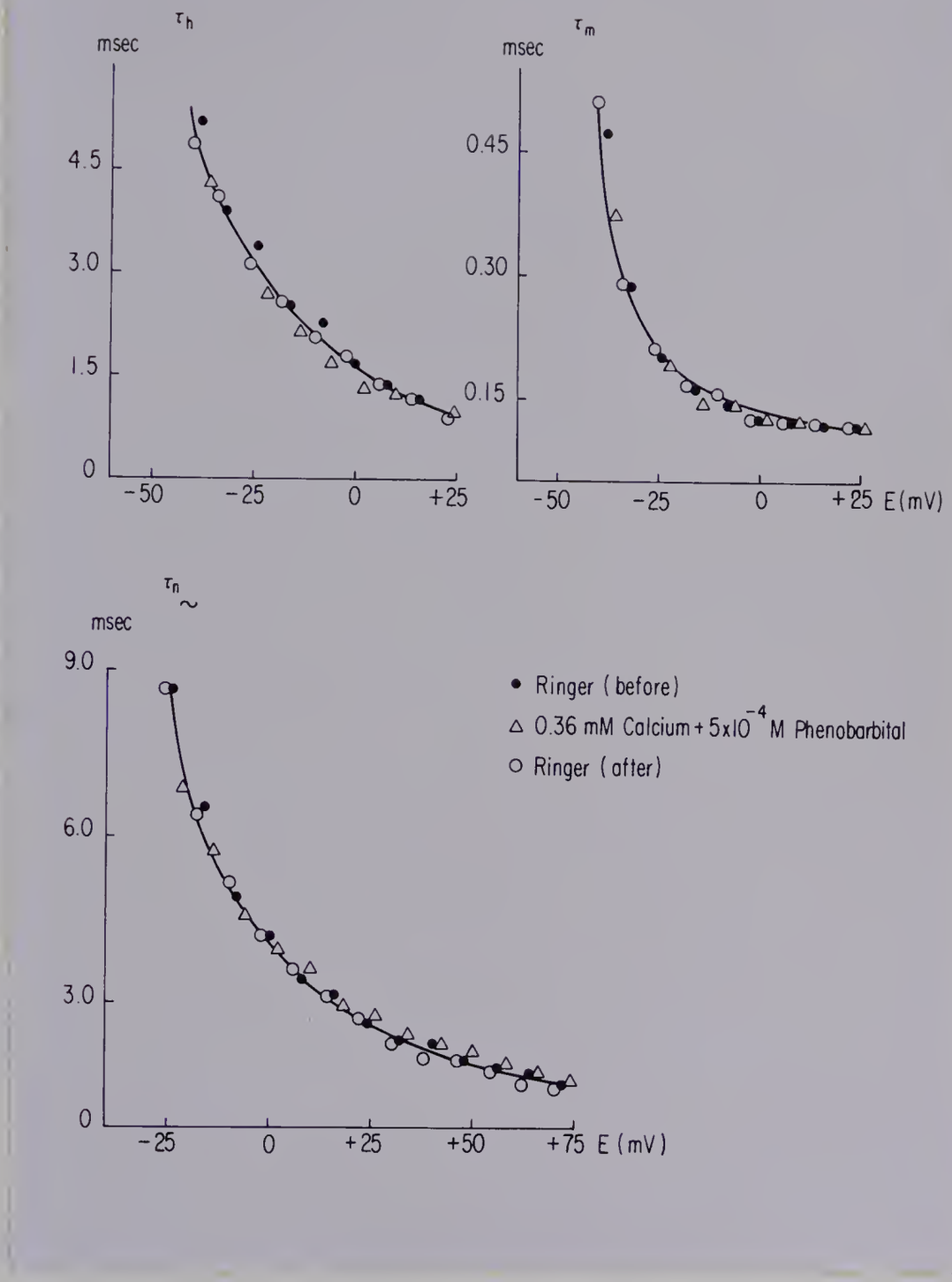


Figure 10. Time constant curves in low calcium phenobarbital Ringer. The sodium and potassium time constant curves in .36 mM calcium plus .5 mM phenobarbital Ringer. Data from the same runs shown for previous figure.

Ringer without drug (not shown) the curves for both T_h and T_m were shifted along the voltage axis to the left by 9 and 10 mV respectively. Thus the shifts expected to occur in low calcium Ringer are abolished by phenobarbital. Lower concentrations of phenobarbital (.1 mM) which did not shift T_m in normal calcium Ringer did not abolish the shifts produced by low calcium.

Three experiments were also performed with 9 mM calcium and .5 mM phenobarbital. In these experiments the reductions in $\bar{g}_{Na}$ were smaller than with phenobarbital alone although the differences when observed were small. When both high calcium and phenobarbital were present T_h and T_m were shifted along the voltage axis to the right by 10 and 15 mV respectively. It appears that the shift in T_m produced by phenobarbital and by different calcium concentrations is additive. T_h on the other hand did not show this type of simple additive behavior.

Although phenobarbital and pentobarbital differ structually in only one side chain on the barbituric acid molecule, their actions on the nodal membrane are in many respects quite different. Figure 11 shows the current-voltage relations before, during and after .5 mM pentobarbital. Both the peak and steady state currents are reversibly reduced in amplitude but in contrast to phenobarbital the steady state currents are reduced to a

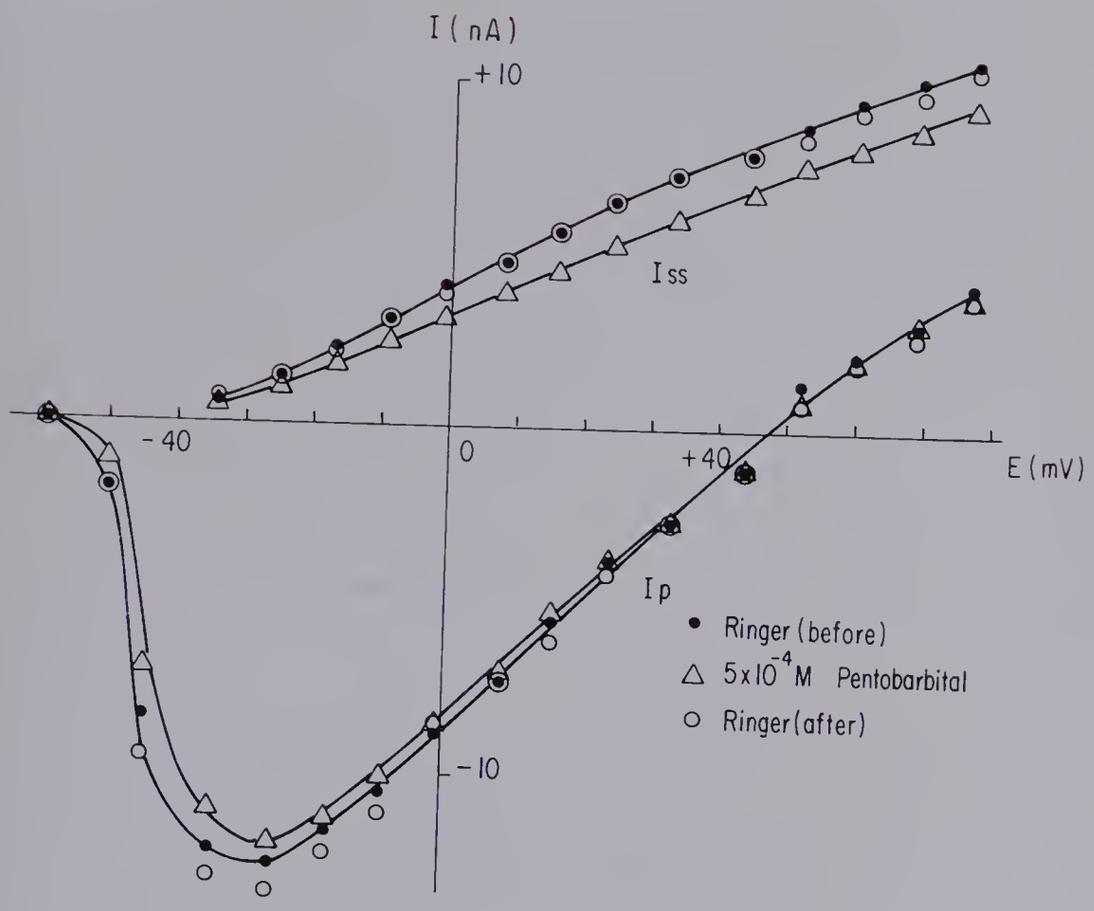


Figure 11. Current-voltage relations in pentobarbital. Peak (Ip) and steady state (Iss) currents in .5 mM pentobarbital. T=6°C. Axon 82

greater extent than the peak currents. As with phenobarbital the smaller currents are due to reductions in $\bar{g}_{Na}$ and $\bar{g}_{K}$. The reductions in $\bar{g}_{K}$ were from 2 to 3 times larger than the corresponding reductions in $\bar{g}_{Na}$. Pentobarbital at a concentration of .5 mM reduces $\bar{g}_{Na}$ and $\bar{g}_{K}$ from 90 tc 95% and 80 to 85% of control values respectively while 1 mM reduces $\bar{g}_{Na}$ and $\bar{g}_{K}$ from 60 to 70% and 55 to 65% of the ccntrols (3 experiments). The leakage conductance, on the other hand, was unaffected at all concentrations of pentobarbital.

Figure 12 shows the sodium and potassium time constant curves obtained using the runs illustrated in the previous figure. As is clear from the figure neither T_m or T_h is affected by .5 mM pentobarbital. With 1 mM pentobarbital T_h and T_m could not be obtained because the currents were tco small tc allow reliable measurements. The time to peak of the inward current was therefore measured and was found nct to vary with pentobarbital treatment. These results may be contrasted with those of Narahashi et al (1969) and Blaustein (1968a) where the time to peak measurements were prolcnged by pentobarbital.

The lower part of Figure 12 shows the shift in the T_n curve along the voltage axis. This shift was always observed with pentobarbital and is probably indicative of a shift in the curve relating n to membrane potential.

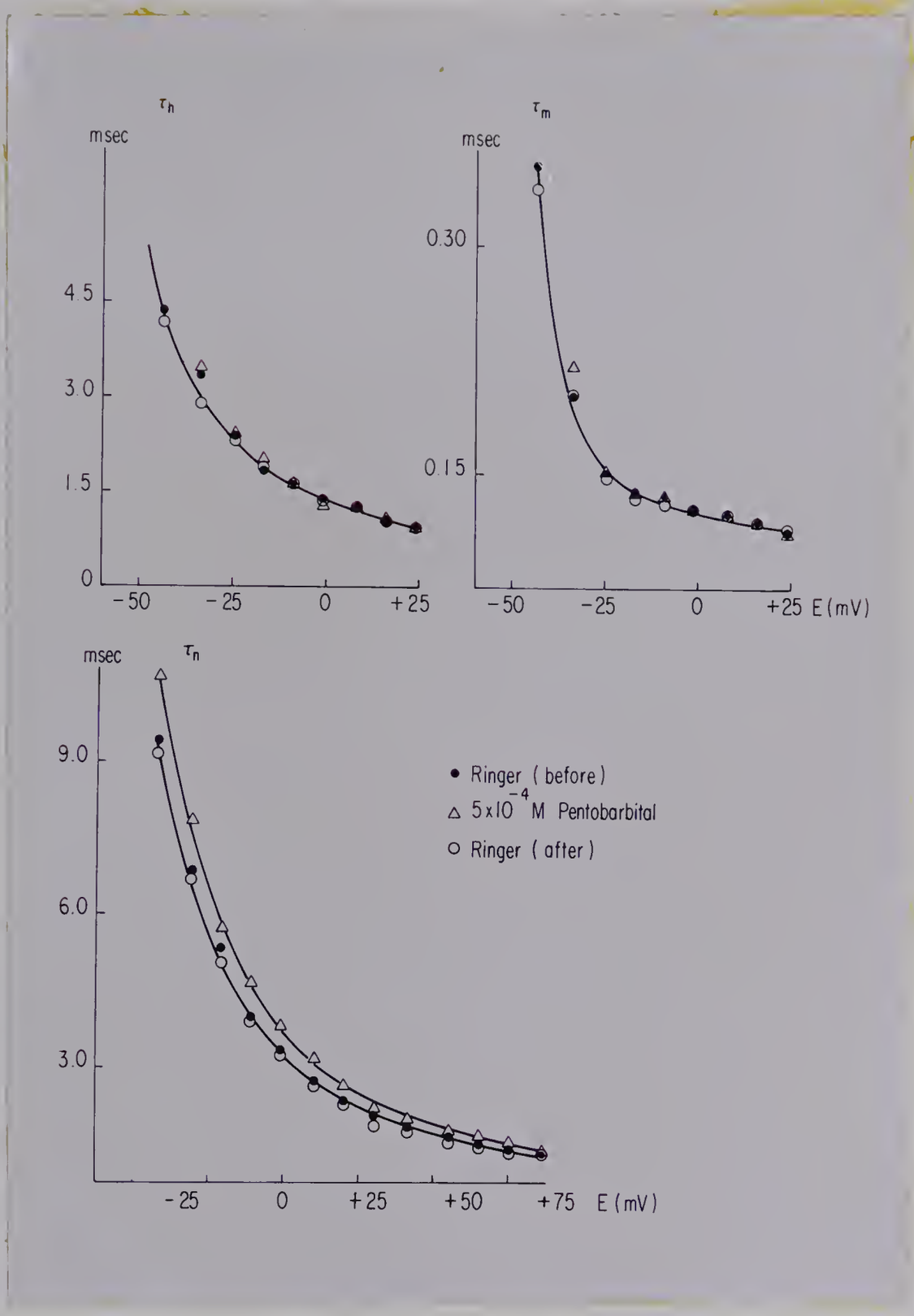


Figure 12. Time constant curves in pentobarbital. Sodium and potassium time constant curves in .5 mM pentobarbital. Data from the same runs shown in the previous figure.

This would prcduce a decrease in the potassium currents over and abcve the reduction caused by a reduced $\bar{g}_K$. In frog skeletal muscle Thesleff (1956) found that low concentraticns of pentobarbital decreased the threshold for action potential production and suggested that it might be due to a decrease in $\bar{g}_K$. My results would support Thesleff's suggestion, but in the frog node pentobarbital does nct produce this effect because it is the level of $\bar{g}_L$ and $\bar{g}_{Na}$ which is mainly responsible for determining threshold and this parameter is affected only to a small extent by $\bar{g}_K$. (Dodge, 1963; Frankenhaeuser , 1965).

Figures 13 and 14 show the results of an experiment in which .5 mM pentobarbital was applied to the node in Ringer containing .36 mM calcium instead of the 1.8 mM calcium. The controls in this case also consisted of .36 mM calcium Ringer. The results in low calcium are similar to those in normal calcium except recovery is not as good and both $\bar{g}_K$ and $\bar{g}_{Na}$ are reduced to a greater extent. The leakage conductance is not affected. As was the case in normal calcium, pentobarbital did not shift either of the curves for T_m or T_h. This shculd be contrasted with Figure 9 in which phencbarbital blocked the shifts normally seen in .36 mM calcium. T_n was shifted to the right by pentcbarbital in .36 mM calcium as it was in 1.8 mM calcium except that the shift was larger. As the

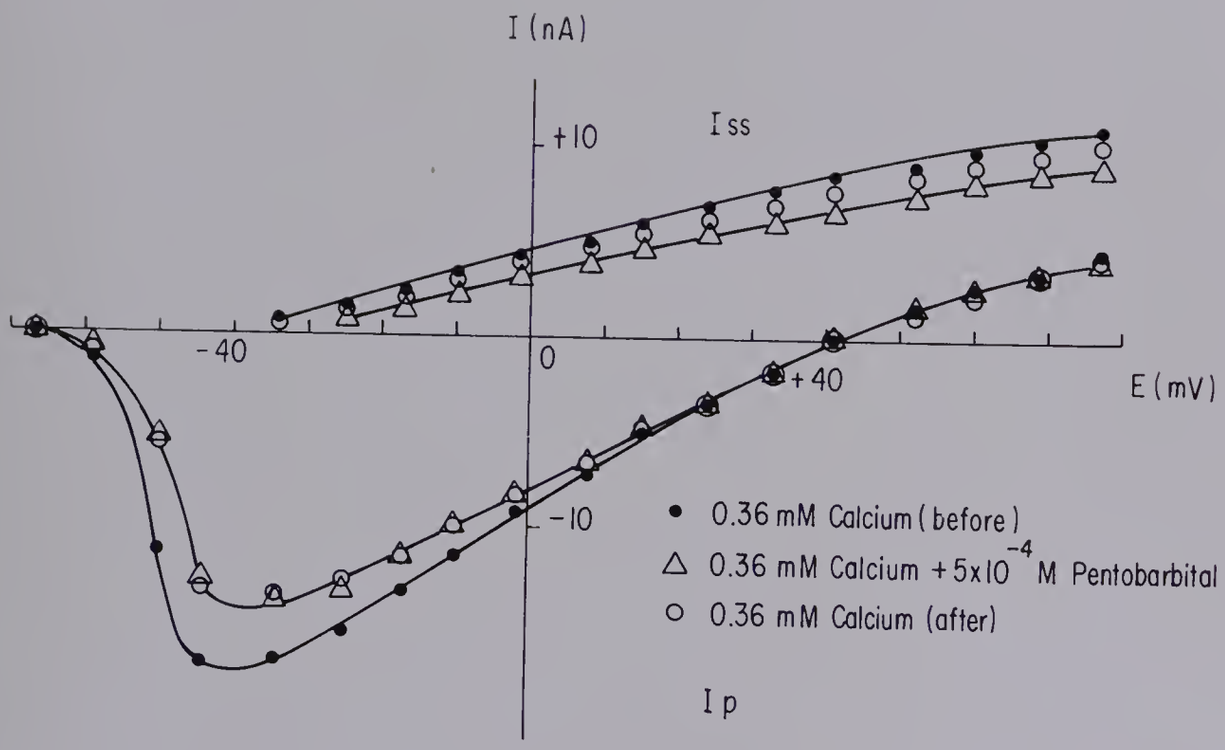


Figure 13. Current-voltage relations in low calcium pentobarbital. Peak (Ip) and steady state (Iss) currents in .5 mM pentobarbital in .36 mM calcium Ringer. T=6°C. Axon 82

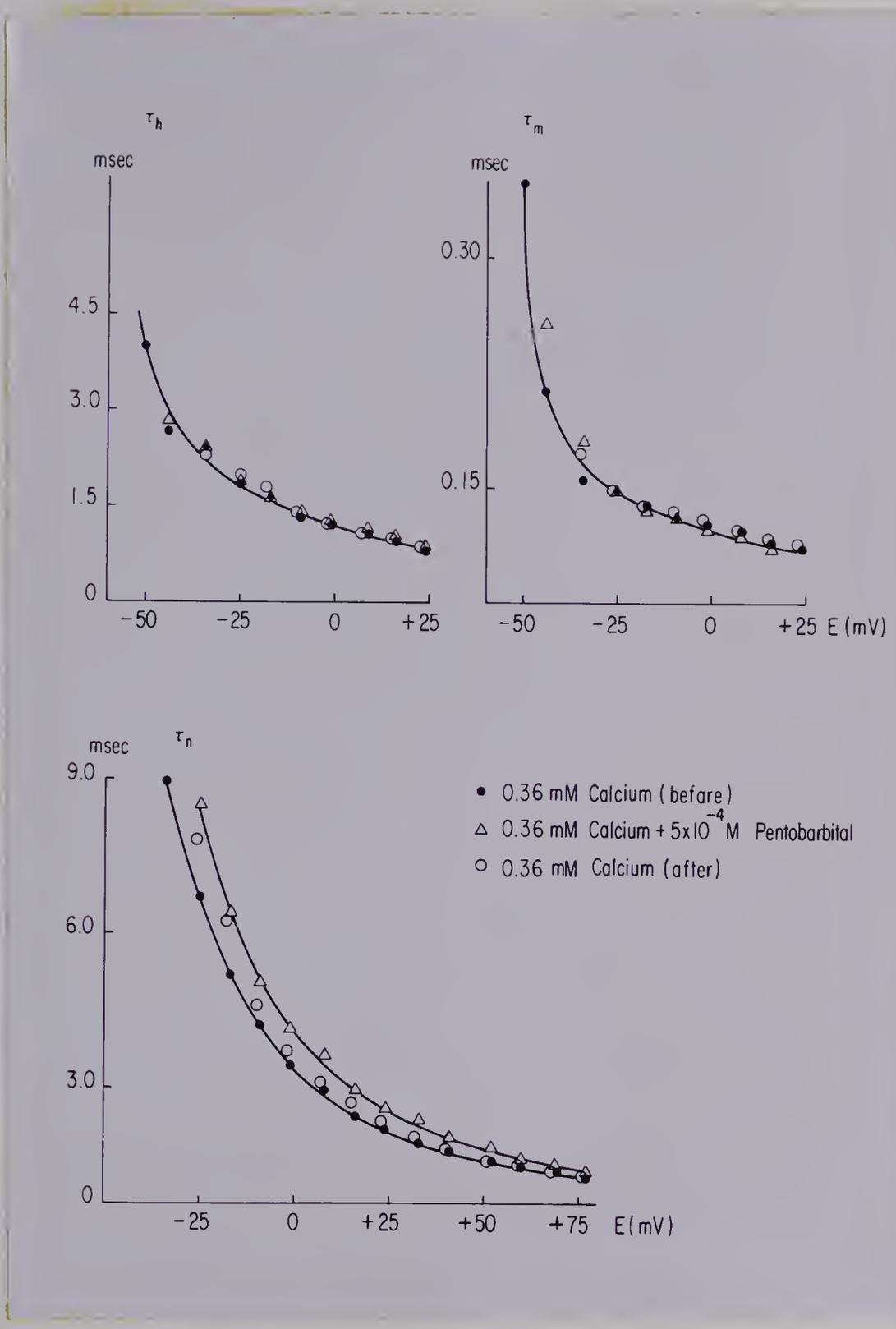


Figure 14. Time constant curves in low calcium pentobarbital. Sodium and potassium time constant curves in .36 mM calcium plus .5 mM pentobarbital Ringer. Data from the same runs shown in the previous figure.

experiments with .36 mM calcium and pentobarbital were carried out on only one axon these results should be regarded as tentative.

In summary, both phenobarbital and pentobarbital reduced $\bar{g}_{Na}$ without affecting the leakage conductance. Pentobarbital also decreased $\bar{g}_K$ and to a greater extent than $\bar{g}_{Na}$; when phenobarbital does decrease $\bar{g}_K$, this decrease is smaller than the simultaneously produced decrease in $\bar{g}_{Na}$. Phenobarbital does not shift T_n nor in most cases T_h but it always shifts T_m. In .36 mM calcium solutions the shifts normally present are abolished by phenobarbital. Pentobarbital on the other hand only produces a shift in T_n and does not abolish the shifts produced in T_m and T_h by low calcium solutions .

Chloral Hydrate and Trichloroethanol (TCE)

Chloral hydrate has effects on the central nervous system similar, in many respects, to the barbiturates and is used clinically for hypnosis and sedation. Although it is effective against experimentally induced convulsions, like most of the barbiturates it has a low ratio of anticonvulsant to sedative activity. (Goodman and Gilman, 1970). Trichloroethanol is one of the breakdown products of chloral hydrate and <u>in vivo</u> is probably the agent responsible for the effects observed when chloral hydrate

is given.

The current-voltage relations for the peak and steady state currents before, during and after .1 mM TCE are shown in Figure 15. As is clear from the figure, both the peak and steady state currents are reduced. The reduction in the steady state currents is due to a reduction in $\bar{g}_K$ which in this figure is reduced to 61% of control. $\bar{g}_{Na}$ also is reduced to 80 % of control but along with this reduction is a pronounced increase in the threshold for inward sodium current flow. The leakage conductance is not affected by TCE. Similar results were obtained with two other nodes.

The sodium and potassium time constant curves obtained from the same experiments are shown in Figures 16 and 17. T_n, T_m and T_h are all shifted by .1 mM TCE. The most interesting feature to note is that while the T_n and T_m curves are shifted in the direction of depolarization, the curve for T_h is shifted in the opposite direction. That is to say, whereas the rate of turning on of the sodium and potassium systems are reduced, the rate of sodium inactivation is increased. This is an unusual finding because it is generally found that conditions which shift T_m usually shift T_h in the same direction along the voltage axis. When a node is current clamped and exposed to .1 mM TCE the recorded action potentials

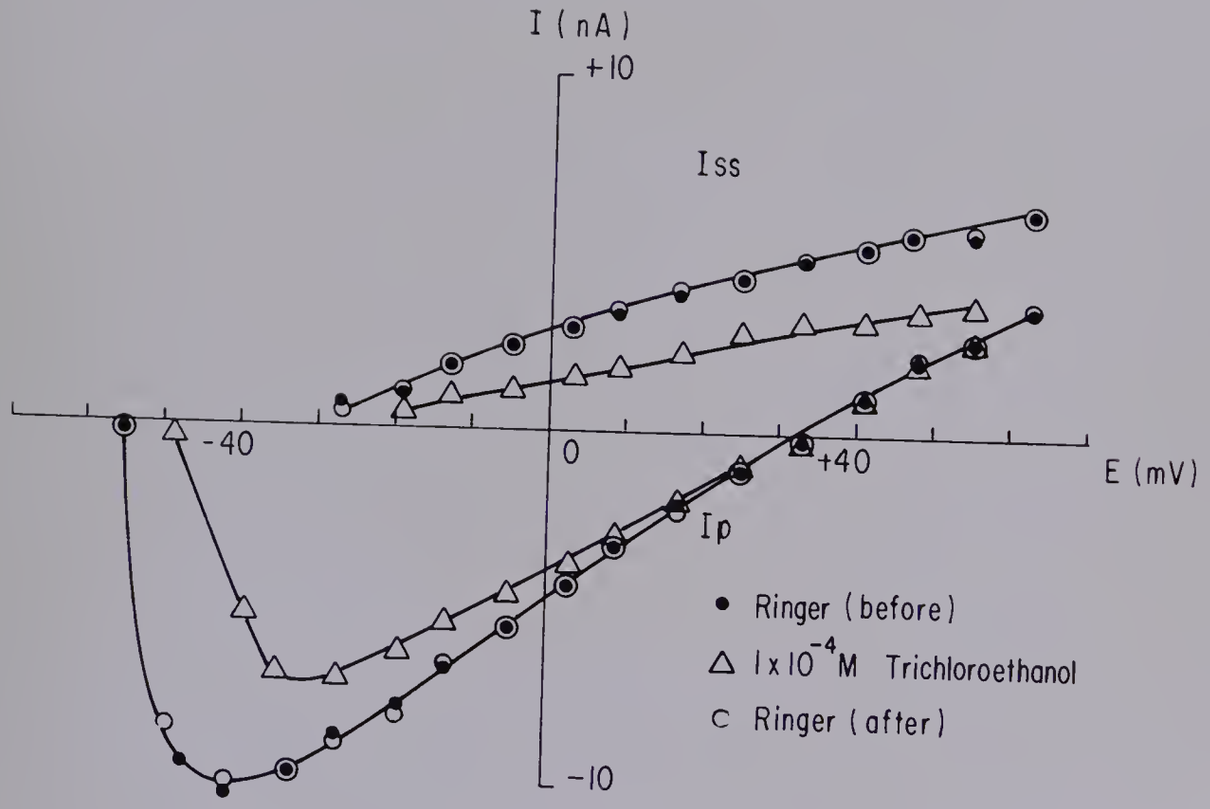


Figure 15. Current-voltage relations in trichloroethanol. Peak (Ip) and steady state (Iss) currents in .1 mM TCE. T=5°C. Axon 68

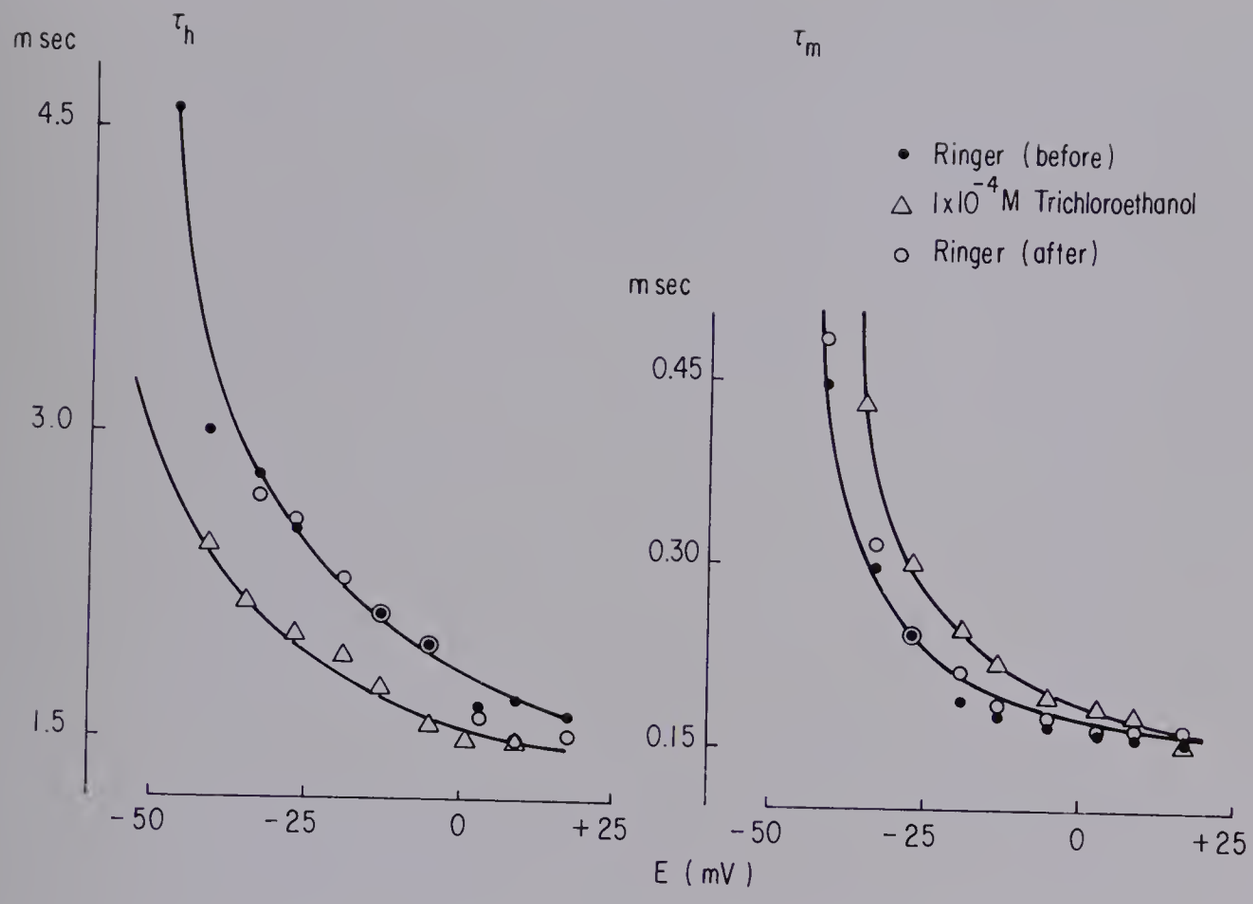


Figure 16. Sodium time constant curves in TCE. T_h and T_m in .1 mM TCE. Data from the same runs shown in the previous figure.

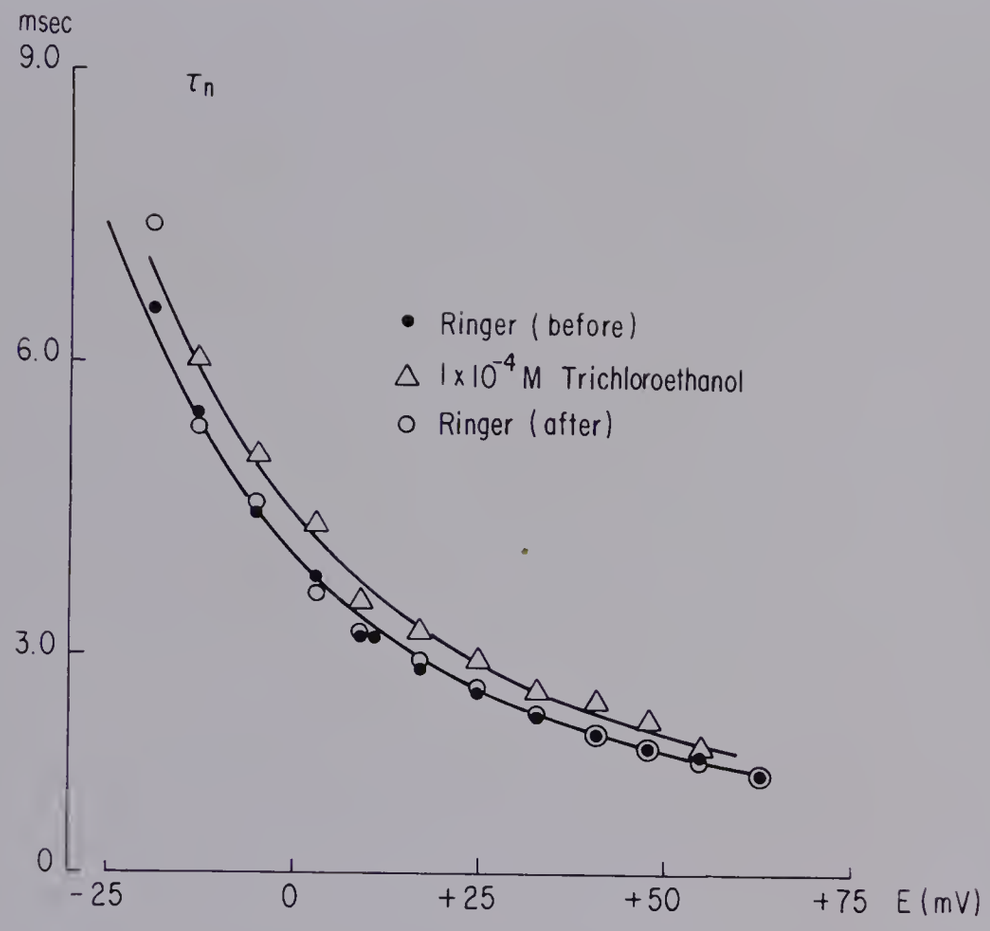


Figure 17. Potassium time constant curve in TCE. Data from same runs shown in Figures 15 and 16.

are reduced in amplitude tc about 80% of control values and show increased thresholds. The reduction in the amplitude of the action pctential cannot be explained by a reduction in $\bar{g}_{Na}$ alone, because this concentration of TCE only reduces $\bar{g}_{Na}$ to between 75 and 80% of control values. It appears that this reduction in amplitude results from a combination cf a decreased $\bar{g}_{Na}$, an increased sodium inactivation, and a decreased sodium activation at small depolarizations.

Chloral hydrate is qualitatively similar to TCE in that it shifts the T_m and T_h curves in the opposite directions along the voltage axis but only at considerably higher concentrations . Using the reduction in $\bar{g}_{Na}$ as a measure of potency, 10 mM chloral hydrate is approximately equivalent to .05 mM TCE in that $\bar{g}_{Na}$ is reduced to about 90% of control (3 experiments). Chloral hydrate has a smaller and a more variable effect on $\bar{g}_K$ than does TCE. Trichloroethanol always reduced $\bar{g}_K$ to a greater extent than $\bar{g}_{Na}$ whereas chlcral hydrate had less affect on $\bar{g}_K$ than $\bar{g}_{Na}$, and in one axcn of three did not reduce $\bar{g}_K$ at all.

The shifts in the T_n curve seen with .1 mM TCE were always in the direction cf depolarization, but with lower concentraticns or with chlcral hydrate the effects on the T_n curve were more variable . In one axon for example, T_n

was shifted to the left by 10 mV, whereas in another axon chloral hydrate failed to produce any shift in T_n.

In order to determine whether calcium would modify the action of TCE, .05 mM TCE was added to the node in either .36 mM or 9 mM calcium Ringer. The different calcium concentrations did not affect the reduction in either $\bar{g}_{Na}$ or $\bar{g}_K$ as compared to the controls containing TCE in 1.8mM calcium Ringer. The curves for both T_m and T_h were shifted along the voltage axis by changing the calcium concentration, but the shifts were simply additive with those produced by TCE alone (2 experiments). For example, the shift to the left of T_h produced by TCE was moved a further 10mV in the same direction when .36 mM calcium and TCE were applied together. Nodes exposed to solutions of .36 mM calcium and TCE were so inactivated that they failed to produce action potentials when the node was current clamped, unless a hyperpolarizing pulse was applied before stimulation.

Although the curve for T_n is not normally affected by .36 mM calcium, the nodes exposed to .36 mM calcium plus TCE showed a greater shift of T_n in the direction of depolarization than they did with TCE in 1.8 mM calcium Ringer. This shift to the right was even apparent in one axon where TCE in 1.8 mM calcium had shifted T_n in the direction of hyperpolarization.

Diphenylhydantoin (DPH)

DPH may be considered as a prototype for antiepileptic drugs as it has a high ratio of antiepileptic to sedative effects (Woodbury et al, 1972). Although a great deal of research has been carried cut in the central nervous system with DPH, only a few studies have investigated the effects on peripheral nerves even though these nerves show some interesting responses to DPH (Toman, 1969).

The current-voltage relations before, during and after .018 mM DPH are shown in Figure 18. As can be seen, only the peak inward current is reduced at this concentration. The smaller currents are due to a decrease in $\bar{g}_{Na}$ to 80% of control. At higher concentrations of DPH the steady state currents were also reduced due to decreases in $\bar{g}_K$. For example, with .18 mM DPH, $\bar{g}_K$ is reduced to 80% of control whereas $\bar{g}_{Na}$ is reduced to 25% of control values (2 experiments). The leakage conductance remained unchanged at all concentraions of DPH up to and including .18 mM.

Because DPH was made up with the commercial solvent (primarily propylene glycol) which is supplied with the drug, control runs were carried out using the solvent

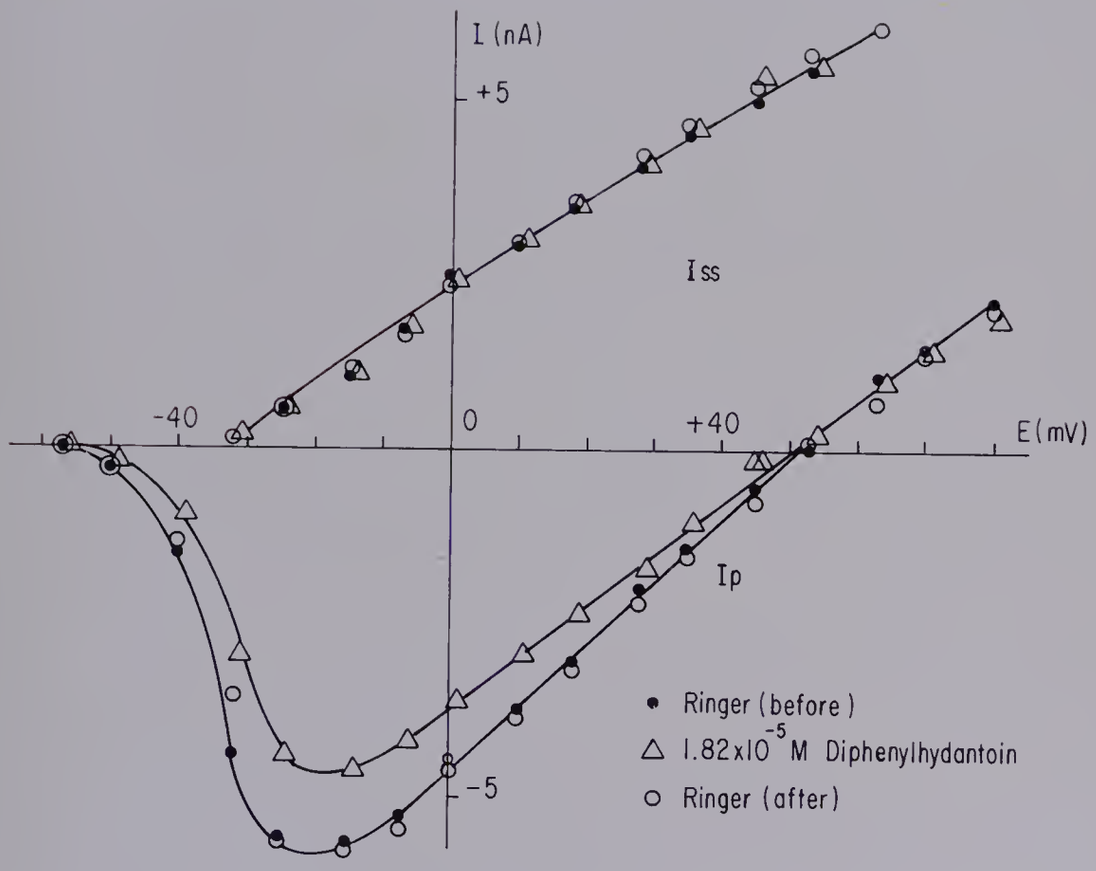


Figure 18. Current-voltage relations in diphenylhydantoin. Peak (Ip) and steady state (Iss) currents in .018 mM DPH. T=6°C Axon 83

alone at three times the concentration used in the experiments with DPH. The data from these runs were found to be no different from those in Ringer without the solvent.

Figure 19 shows the current-voltage relations for .018 mM DPH in .36 mM calcium Ringer. As with DPH in normal calcium the steady state currents are not affected. Once again the peak currents are reduced by a decrease in $\bar{g}_{Na}$ to 78% of control. The poor recovery after drug treatment in .36 mM calcium was usually observed with axons treated with DPH (3 experiments).

The effects on the sodium time constant curves by DPH and DPH in .36 mM calcium are shown in Figure 20. The controls in the top of Figure 20 are 1.8 mM calcium Ringer while those in the bottom of the figure are 1.8 mM and .36 mM calcium Ringer . DPH in the presence of 1.8 mM calcium does not effect either T_m or T_h. However, when the calcium concentration is reduced to .36 mM, DPH reduces the normal 10 mV shifts occuring in .36 mM calcium by half. Similar results were obtained with .009 mM DPH in the presence of .36 mM calcium but in this case the normal 10 mV shift was reduced to 7 mV. Higher concentrations of DPH (.036 mM) in 1.8 mM calcium Ringer shifted T_m to the right by 10 mV but did not further affect T_h.

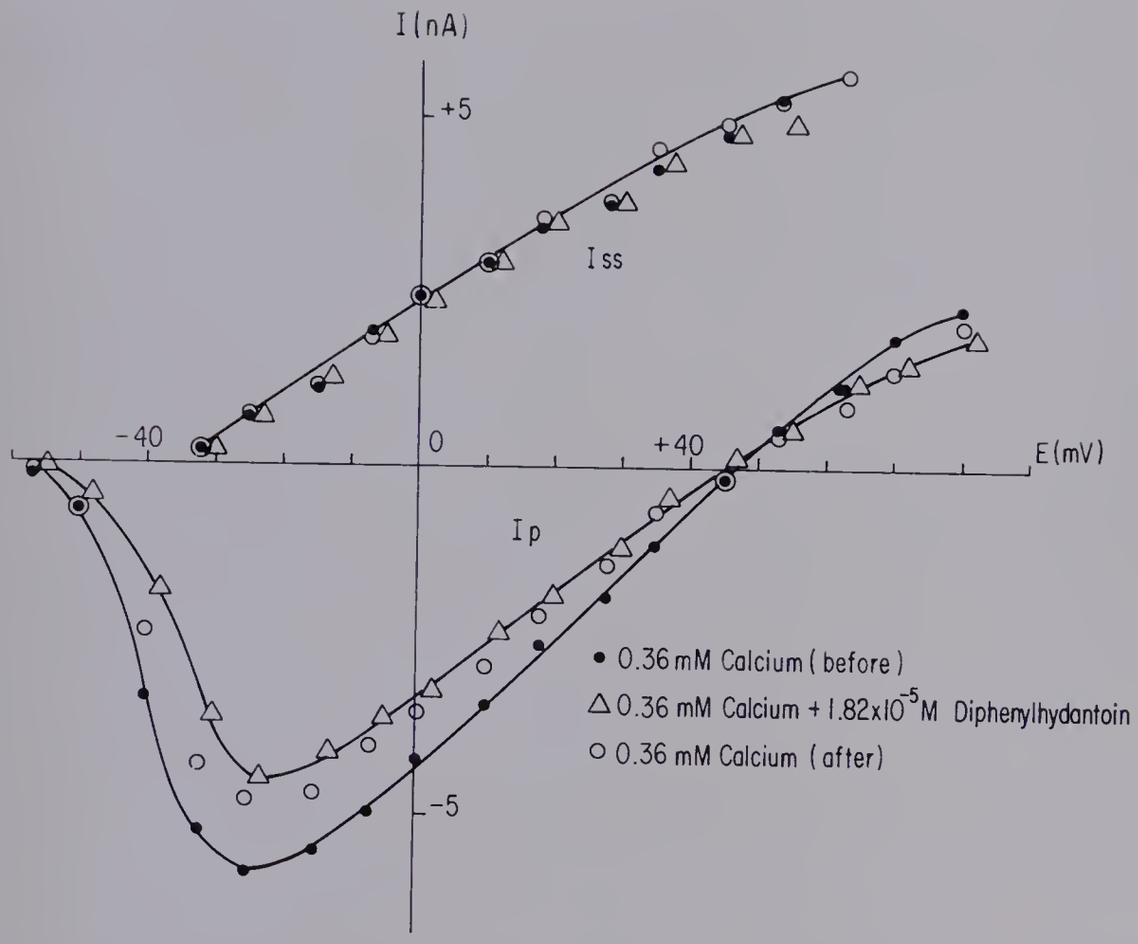


Figure 19. Current-voltage relations for DPH in low calcium Ringer. Peak (Ip) and steady state (Iss) currents for DPH in .36 mM calcium Ringer. T=6°C. Axon 83

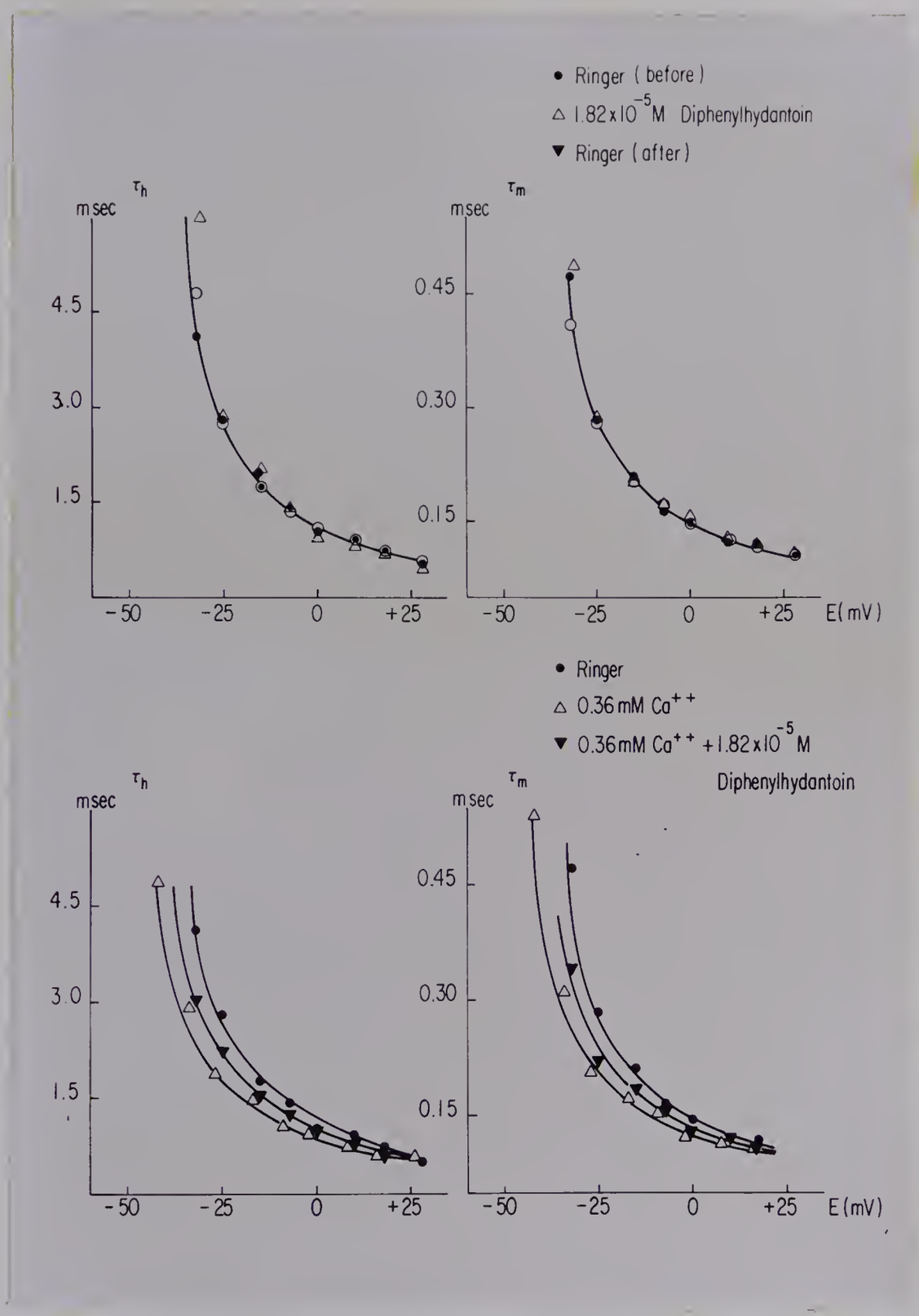


Figure 20. Sodium time constant curves. The upper curves are for DPH in Ringer. The lower curves show the Ringer control (before), the .36 mM calcium control (before), and the test curves with .018 mM DPH in .36 mM calcium Ringer. T=6°C. Axon 83

The time constant curves for the potassium system obtained from the runs shown in the previous figure are shown in Figure 21. It should be noted that the controls at the top of this figure are 1.8 mM calcium Ringer while those at the bottom are .36 mM calcium Ringer. As can be seen from the controls the curve for T_n is not affected by .36 mM calcium. In the presence of .018 mM DPH and .36 mM calcium however, T_n is shifted to the right. This result was atypical, as in the other axons studied neither DPH by itself or in the presence of .36 mM calcium shifted T_n, even at concentrations up to .18 mM.

Chlorpromazine

Chlorpromazine effects have been tested on squid giant axons and it has been found to block both the peak and the steady state currents (Gruener and Narahashi, 1972). Hille (1966, 1967a) found that prochlorperazine, a close relative of chlorpromazine, selectivily reduced $\bar{g}_{Na}$.

Figure 22 shows the current-voltage relations for the peak and steady state currents for Ringer alone, and Ringer containing .005 mM and .01 mM chlorpromazine. A voltage clamp run in Ringer was made after each concentration of drug had been tested but these are not shown because they were identical to the drug series . That is, there was little or no recovery after

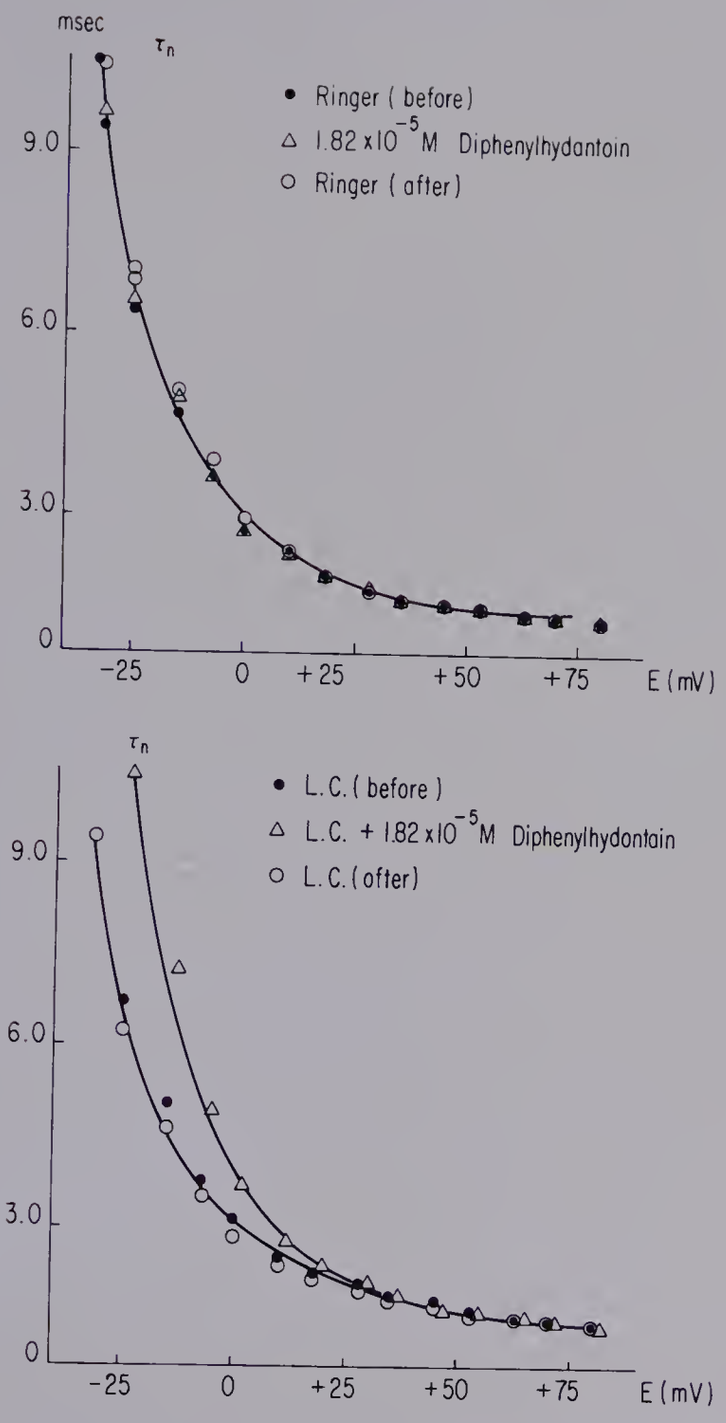


Figure 21. Potassium time constant curves. Upper curves are for DPH in Ringer. Lower curves are for DPH in .36 mM calcium Ringer. Note lower controls are both .36 mM calcium Ringer. T=6°C. Axon 83

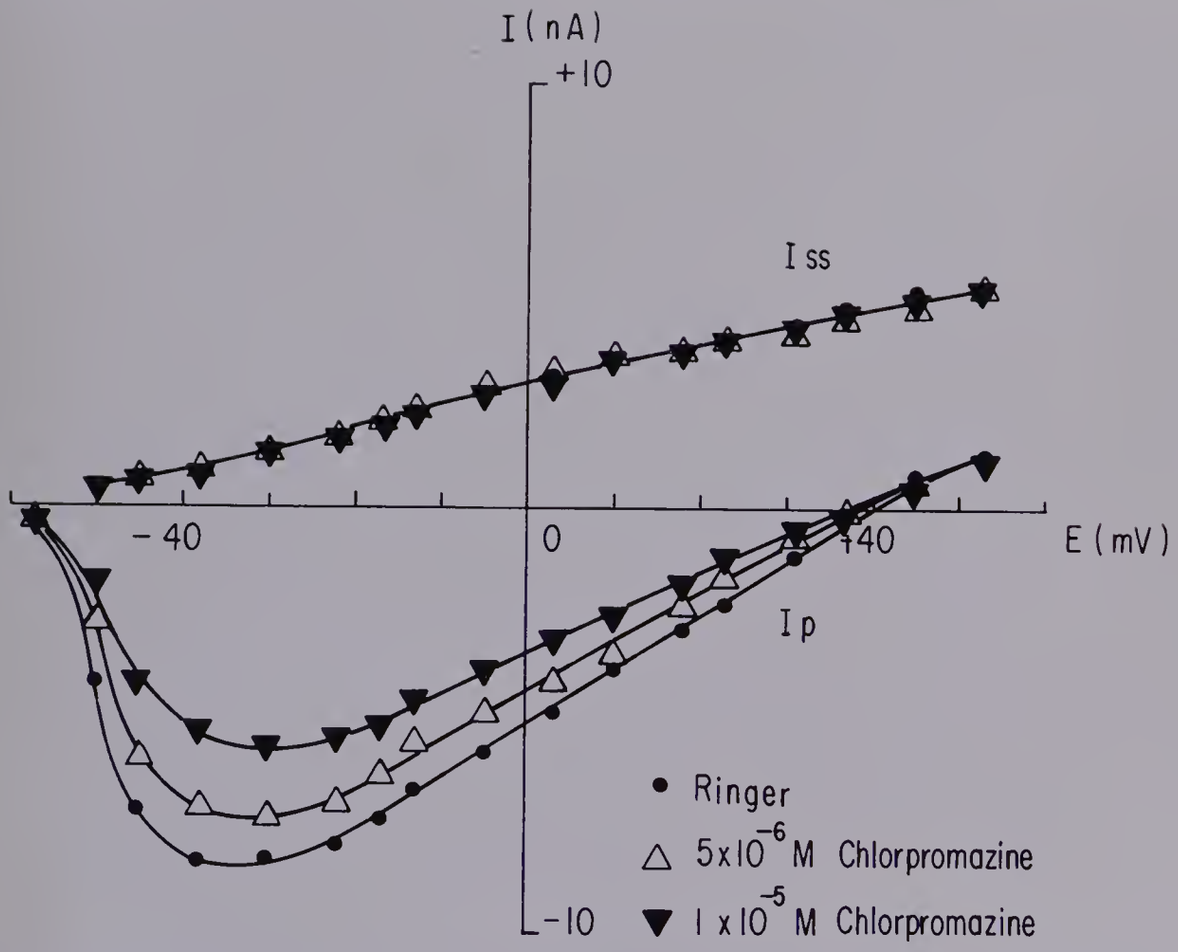


Figure 22. Current-voltage relations in chlorpromazine. Peak (Ip) and steady state (Iss) currents in Ringer (before) and .005 and .01 mM chlorpromazine. T=4°C. Axon 76

chlorpromazine. The prolonged binding of chlorpromazine is perhaps the most interesting difference between chlorpromazine and the other drugs tested in the present study. With the exception of strychnine, the other drugs tested were washed out almost completely within three minutes of returning the node to Ringer. This persistent binding, as indicated by the poor recovery in Figure 21, has also been reported in squid axons (Gruener and Narahashi, 1972). With the frog node, washing for as long as 30 minutes was necessary to effect partial recovery, although in two of five nodes even partial recovery was not obtained.

As can be seen in Figure 22 only the peak transient current was affected by chlorpromazine. This resulted from a decrease in $\bar{g}_{Na}$ which for .005 mM and .01 mM chlorpromazine was reduced respectivity to 85 and 65% of the Ringer control. At still higher concentrations (.05 mM), $\bar{g}_K$ was also reduced but always to a smaller extent than $\bar{g}_{Na}$. Figure 23 shows the sodium and potassium time constants for the same voltage clamp series illustrated in the previous figure. As can be seen there was no effect on any of the time constant curves by chlorpromazine. Similar results were obtained in two other axons in which the time constants were measured.

Chlorpromazine was also tested with Ringer containing

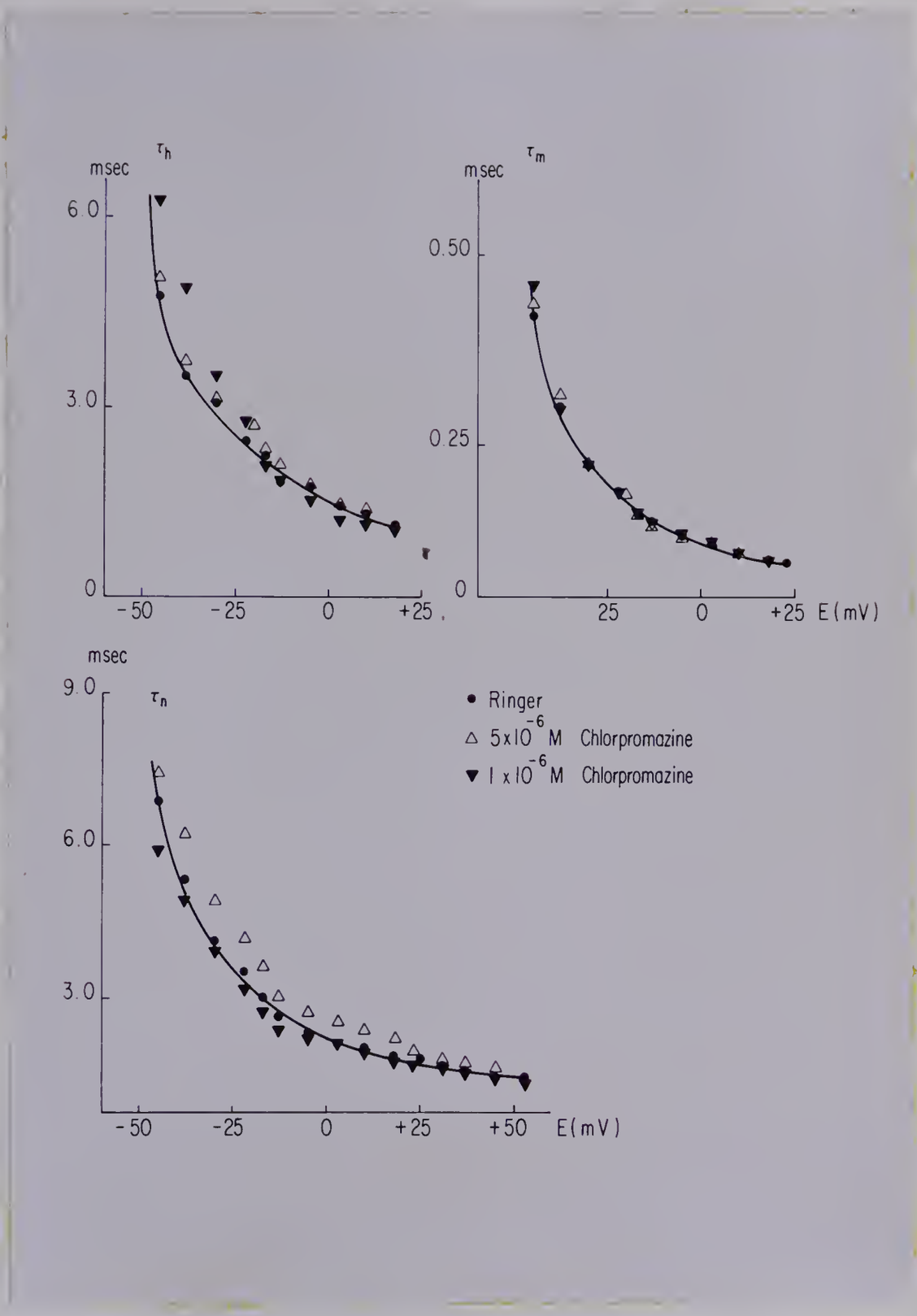


Figure 23. Time constant curves in chlorpromazine. Sodium and potassium time constant curves in .005 and .01 mM chlcrpromazine. Data from same runs shown in the previous figure.

9 mM and .36 mM calcium tc determine whether altering the calcium concentration would modify the action of chlorpromazine. These experiments were rather difficult to carry out because of the poor recovery from the effects of chlorprcmazine. In one axon .005 mM chlorpromazine in 1.8 mM calcium Ringer reduced $\bar{g}_{Na}$ tc 85% of control with no effect on $\bar{g}_{K}$, whereas the same concentration of chlorpromazine in .36 mM calcium Ringer reduced $\bar{g}_{Na}$ to 14% of control and $\bar{g}_{K}$ to 60% cf control. Recovery however, was extremely poor. In two other experiments in which lower concentrations cf chlcrpromazine were employed, no differences were found between the effects produced by chlorpromazine Ringer and .36 mM calcium chlorpromazine Ringer.

Strychnine

Because some of the drugs I have tested are useful as anticonvulsants as well as antiepileptics, I also tested strychnine which is capable of producing convulsions and is sometimes employed in tests for antiepileptic drugs (Woodbury, 1969). Strychnine has been shown to produce prolonged action potentials at the frog node (Maruhashi et al, 1956). Recently Klee (Klee et al, 1973), found that in Aplysia neurons strychnine reduces both the inward and outward currents and reduces the threshold for inward current flow.

In my experiments strychnine reduced both the peak and steady state currents. The most interesting and unusual observation however was an apparent inactivation of the potassium system produced by strychnine. This is illustraded in Figure 24 which shows a voltage clamp series run in the presence of .1 mM strychnine Ringer. For clarity, cnly every cther current record is shown starting at a depolarization of -43 mV and extending to +147 mV. This figure should be compared with the total current record shown in Figure 2. which was obtained in Ringer solutien. It should be noted that the voltage steps used in Figure 24 are much larger than those of Figure 2. As is clear from Figure 24 the inward scdium currents are entirely abolished by treatment with .1 mM strychnine. In this example $\bar{g}_K$ at the peak of the potassium current is reduced to 60% of control while the potassium current at 15 milliseconds is reduced even more. In some axons the initial peak in I_K followed by a decline was apparent at concentratiens of strychnine as low as .01 mM. In two axens out of five studied with strychnine the peaking of I_K fcllcwed by a decline was not observed at concentrations up to and including .1 mM strychnine.

Normally the nodal membrane does not show potassium inactivation unless test pulses are applied longer than 200 mSec. (Frankenhaeuser, 1962). When long test pulses

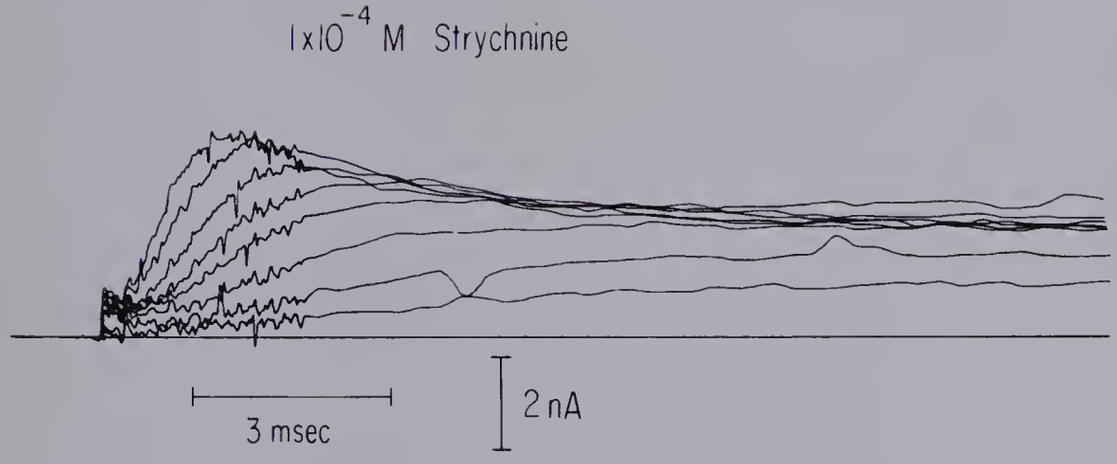


Figure 24. Voltage clamp currents in strychnine. Only every other record is shown, starting at -43 mV and extending to +147 mV. T=4°C. Axon 76

are applied to the node the potassium current declines with time to a final non-zero steady state value which is voltage dependent (Schwartz and Vogel, 1971). The currents in Figure 23 show a similar type of behavior except that the inactivation rate is measured in milliseconds instead of seconds.

Measurements of T_n revealed that it is not affected by strychnine at concentrations up to .1 mM. T_m and T_h were not affected by strychnine. In axons where T_h and T_m were not measured the normalized peak sodium conductance versus membrane potential curves showed no shift which indicate that T_m was not affected.

In current clamp experiments strychnine modified the action potential by reducing the height and prolonging the duration. Concentrations of strychnine between .1 and .5 mM prolonged the action potential duration two to three times the duration in Ringer without strychnine. When .5 mM strychnine was added to the node, the duration was gradually increased while the height decreased until the action potential was completely blocked in two to three minutes.

Other Drugs

Other drugs studied included procaine,

diphenhydramine and sodium bromide. My experiments with procaine gave similar results to the data from squid or node obtained by others with procaine (Shanes et al, 1959; Hille, 1966, 1967a). Procaine reduced the early peak current by reducing $\bar{g}_{Na}$ with little effect on $\bar{g}_K$ and no effect on $\bar{g}_L$. Shifts in the peak sodium conductance along the voltage axis was seen with procaine but the shifts amounted to only a few mV.

Experiments with diphenhydramine were similar to those with procaine except $\bar{g}_K$ was reduced along with $\bar{g}_{Na}$. Diphenhydramine at a concentration of .1 mM reduced $\bar{g}_{Na}$ and $\bar{g}_K$ to 40 and 58% of control values respectively whereas $\bar{g}_L$ was not effected. Shifts in the time constants were either absent or shifted by 2 or 3 mV in the direction of depolarization.

Sodium bromide was also tested on the node but was found to have no effects. Sodium bromide was used instead of sodium chloride to make up the Ringer solution so the concentration of bromide was 111.8 mM. In two experiments the bromide Ringer showed no detectable effects on the node after 30 minutes exposure.

Chapter 5

DISCUSSION

Membrane Stabilization by Narcotics

The one common feature apparent among the narcotics tested in this thesis was their ability to reduce $\bar{g}_{Na}$ and, with two exceptions (to be discussed below), this is the mechanism by which they are able to block the action potential. These findings are in agreement with other studies on nerve and muscle where the consistent results have been that all the narcotics tested thus far reduce $\bar{g}_{Na}$ with or without effects on $\bar{g}_{K}$ or the sodium or potassium kinetics (Frank, 1972; Hille, 1967a; Narahashi, 1971; Narahashi et al, 1969).

Whether these narcotics all reduced $\bar{g}_{Na}$ by the same mechanism cannot be ascertained with certainty from my experiments. One of the difficulties in intrepreting the action of the various narcotics is that at first glance they do not appear to act via classical pharmacological receptors. This is clearly illustrated by the observation that widely divergent classes of compounds such as detergents, tranquillizers and local anesthetics can produce a reduction in $\bar{g}_{Na}$ (Hille, 1967a; Kishimoto and Adelman, 1964; Narahashi et al, 1969). This lack of

specificity has resulted in a great deal of speculation concerning a unified physical theory of narcosis by which this action may be attributed to some physical change in the membrane structure. This could explain why different types of drugs can all produce the same effects (Seeman, 1966, 1972). One suggested modification of membrane structure by narcotics has been a 'fluidization' of the membrane. The molecules of the narcotic supposedly dissolve in the membrane lipids causing a disordering of the lipids and producing membrane expansion. This expansion has been shown to take place in a variety of membranes and accounts for the protection of red cell membranes to osmotic lysis conferred by low concentrations of narcotics and the lysis which ultimately occurs when the drug concentration is raised (Seeman, 1972). It has been suggested that this membrane expansion might alter the conformation of the membrane in such a way that the sodium channel is affected and $\bar{g}_{Na}$ reduced. It should be noted in this regard that the concentration-dependent expansion of red blood cell membranes by narcotics correlates quite well with the concentration of the narcotic necessary to block nerve fibre conduction (Seeman,1972). In some respects this model is similar to that of Shanes (1958b) in which he suggested that the narcotic molecules dissolve in the membrane and produce a lateral pressure within the membrane which interferes with the increased permeability to sodium necessary for

excitation. This suggestion finds support in Skou's work in which he found that the potency of various local anesthetics was related tc the degree to which they could increase the surface pressure of lipid monolayers (Skou, 1961). The finding that increased hydrostastic pressure can reverse anesthesia is also in agreement with this suggestion (Jchnscn et al, 1954).

There are however, several lines of evidence which argue against such a uniform physical mechanism for all narcotics, although it may operate in the case of certain compounds. First, perfusion studies have demcnstrated that some drugs, such as the local anesthetics and pentobarbital, are more effective when administered to one side of the membrane than the other, suggesting that there is a specific site at which these drugs interact to produce their effects. Narahashi and his coworkers have found that the local anesthetics act in the charged form on the internal surface of the membrane whereas pentobarbital acts in the uncharged form also at the internal surface (Narahashi et al, 1970; Narahashi and Frazier, 1971).

Second, there are drugs such as tetrodotoxin which are not soluble in lipids and act at a specific receptor site to reduce only $\bar{g}_{Na}$ (Hille, 1967a, 1968a; Narahashi et al, 1967). This suggests that at least one specific

receptor site exists which is capable of reducing $\bar{g}_{Na}$ without producing fluidization of the membrane.

Third, the effects of chlorpromazine on the action potential of the squid axon are very sensitive to the pH of the bathing medium. When an axon is exposed to chlorpromazine at pH 8.8 the action potential is gradually blocked. Perfusing the axon with drug free solution at pH 8.8 will, within a few minutes, restore the action potential to its full height. Now if the solution pH is changed to 7.6 the action potential is again blocked. Returning the pH to 8.8 once again restores the action potential to its original height (Gruener and Narahashi, 1972). Since the pKa of chlorpromazine is somewhere near 8.0 the effect of changing the pH is to convert chlorpromazine from the cationic to the neutral form. This clearly demonstrates that it is not simply the presence of the drug in the membrane which is important for supressing $\bar{g}_{Na}$ but also the form that the drug assumes within the membrane. A similar effect also has been shown with pentobarbital (Blaustein, 1968) and some local anesthetics (Ritchie et al, 1965).

Fourth, recent experiments investigating the action of quaternary analogs of lidocaine on the internal surface of frog nodes have shown a voltage dependent reduction of $\bar{g}_{Na}$ by these drugs (Strichartz, 1973). Both a "tonic" and

a "phasic" block are produced by these drugs. The "tonic" block only takes effect after the membrane has been depolarized sufficiently tc raise m to a value of one. Once this "tonic" phase has been produced, the block is no longer sensitive tc variations in the membrane potential. There is however a "phasic" block which remains voltage-sensitive and can be increased or decreased depending on the previous voltage conditioning of the membrane. These observations indicate that the blockade of the sodium system requires either a change in the state of the sodium channel or exposure of a site when the sodium system is activated; this site is presumably where drugs bind to produce their effects.

Fifth, the most noticeable feature of the narcotics tested in this thesis and and of those reported in the literature, in addition to their ability to reduce $\bar{g}_{Na}$ is the variability of their other effects (Narahashi, 1971). For example, phenobarbital had little effect on $\bar{g}_K$ and shifts the curve for T_m in the direction of depolarization, whereas pentobarbital did not effect T_m but had a large effect on $\bar{g}_K$ and shifted T_n to the right. On the other hand chlorpromazine only reduced $\bar{g}_{Na}$ with little or no effect on $\bar{g}_K$ and no effect on any of the time constants. It seems rather improbable that the sodium channel lacks specificity whereas the potassium system and the kinetic parameters for the sodium and potassium

systems show rather a high degree of drug selectivity. Taken together the above considerations would suggest that there is probably no single mechanism which can account for the reduction in $\bar{g}_{Na}$ observed with the different narcotics.

Chloral hydrate and trichlororthanol, unlike the other narcotics , reduce the action potential by mechanisms other than a simple reduction in $\bar{g}_{Na}$. In general action potentials may be reduced in at least three ways; 1) a reduction in $\bar{g}_{Na}$; 2) a shift in the curve relating $\underline{h}$ to membrane potential in such a way that the sodium system is inactivated to a greater extent at the resting potential and 3) $\underline{m}$ is shifted in such a way that the threshold is greatly increased. With trichloroethanol and chloral hydrate all three of these processes are operative and each contributes to the reduction in the action potential. Only in one study with narcotics has it been suggested that sodium inactivation might be the mechanism for producing blockade of the action potential. Schoepfle (1957) concluded that thiopental, when added to the frog node, blocked the action potential by increasing sodium inactivation because the block could be reversed by a preceeding hyperpolarization. As Blaustein (1968) pointed out however, Schoepfle also claimed that the intrinsic time of the inactivation process was not modified. This clearly does not fit with the model

presented in Chapter 1 of this thesis, as any change in $\underline{h}$ would be reflected by a change in the relation between T_h and the membrane potential. The other difficulty with Schoepfle's explanation is that even if only $\bar{g}_{Na}$ were reduced, a prehyperpolarization would certainly increase the height of the action potential, because the sodium system would be less inactivated and therefore the sodium current would be expected to increase. This reversal of action potential blockade with hyperpolarization has been shown to occur with procaine which does not effect sodium inactivation and which itself hyperpolarizes the membrane by a few millivolts (Inoue and Frank, 1965).

The fact that T_h can be shifted in the direction opposite to that of T_m and T_n by chloral hydrate and trichloroethanol indicates that the kinetic parameters are determined by distinct processes. In contrast, it has been suggested that sodium activation, sodium inactivation, and potassium activation are coupled processes (Goldman, 1964; Weiss, 1969) or, in a more restricted model, that sodium activation and inactivation were coupled but that potassium activation was a seperate process (Hoyt, 1963; Hoyt and Adelman, 1970; Hoyt and Strieb, 1971). However, studies with pharmacological agents such as tetraethylammonium and tetrodotoxin have shown that a coupled process between the sodium and potassium system is highly unlikely (Hille, 1967a, 1967b,

1970). Indeed, Chandler et al (1965) attempted to determine whether activation and inactivation in the sodium system were coupled, and concluded that they were sepearate processes. The experiments in this thesis with trichloroethanol and chloral hydrate clearly demonstrate that the processes described by m and h are distinct because the rate at which the sodium system turns on, is decreased under conditions where the rate of turning off the sodium system is increased. It is rather difficult to imagine that m and h are coupled processes when drugs can shift these parameters in opposite directions.

It is interesting to note that the mechanism of nerve block by chloral hydrate is so different from that of the barbiturates, and yet their effects on the central nervous system are so similar. I can only speculate from my data, but it seems likely that it is a reduction in the sodium system, no matter how it is achieved, which is essential for general central nervous system depression . The fact that the drug concentrations used in this thesis are higher than those necessary to produce central nervous system depression does not argue against a similar mechanism of action, but demonstrates that motor nerves are less sensitive to narcotics than are central neurons. This conclusion is in agreement with the work of Frank and his coworkers showing that a wide variety of drugs which reduce the sodium mechanism all have depressant properties

in the central nervous system (Frank, 1972).

Stabilization by Antiepileptic Drugs

Antiepileptic drugs may or may not supress excitability, and many narcotics are not useful as antiepileptics. A main characteristic of antiepileptic drugs used for the treatment of grand mal epilepsy is their ability to supress spontaneous and repetitive activity in nerves made hyperexcitable by various procedures (Toman, 1969).

Spontaneous activity occurs in nerve when there is a net inward flow of current at the resting membrane potential (Noble, 1966). In the squid axon repetitive activity may be produced by applying a steady depolarizing current to the membrane, and spontaneous activity by reducing the calcium and magnesium concentration in the bathing medium. The steady depolarization produces repetitive activity because the membrane is held at a potential at which $\bar{g}_{Na} m^3 h (E-E_{Na})$ is greater in magnitude than the sum of the leakage and potassium currents so there is a net inward flow of current (Woodbury, 1969). Huxley (cited by Frankenhaeuser and Hodgkin, 1957) suggested that a reduction in extracellular calcium concentration might alter the sodium and potassium kinetics by reducing the potential gradient across the

membrane without changing the measured resting membrane potential. If calcium is normally bound to fixed negatively charged sites on the external surface of the membrane and therefore acts to neutralize these charges, then removal of part of this bound calcium by reducing the calcium in the bathing solution would have the effect of decreasing the potential gradient across the membrane. Since it is likely that the voltage-dependent parameters are dependent on the potential gradient across the membrane, and not the resting membrane potential as normally measured, this would account for the apparent shifts in the curves relating m and h to membrane potential. A similar type of effect is observed when axons are perfused with solutions of low ionic strength (Chandler et al, 1965). This would explain why reduction in the calcium concentration is equilivalent in most respects, but not all (Frankenhaeuser and Hodgkin, 1957), to reduction of the membrane potential. The effects on excitability produced by elevating the extracellular calcium concentration would also be explained by this mechanism. In this instance, an increased binding of calcium to the negative fixed charges of the membrane would create a larger potential gradient across the membrane and produce an effect similar to hyperpolarization.

Antiepileptic drugs might therefore reduce

spontaneous activity, by any mechanism which would reduce $\bar{g}_{Na} m^3 h (E-E_{Na})$, to a value less than the sum of the leakage and potassium currents. From my experiments it appears that the parameter modified by drugs which block spontaneous firing is m. The degree of sodium activation is not increased at the 'resting potential', resulting in a stable 'resting' condition and thereby preventing oscillations in the membrane potential. Although T_h and $\bar{g}_{Na}$ also were affected by treatment with the antiepileptics, the reductions in $\bar{g}_{Na}$ were rather small. Further, in the .36 mM calcium solutions containing drug, T_h was affected in such a way that the sodium system would be less inactivated and thus favor increased activity. It should be noted that the concentrations necesary to produce these effects, at least for diphenylhydantoin, are in the same range as the plasma concentrations necessary to control grand mal epilepsy (Woodbury et al, 1970). This suggests that diphenylhydantoin may prevent epileptic seizures in the central nervous system by a mechanism similar to that found for the nerves studied in this thesis.

It is interesting that in 1.8 mM calcium both phenobarbital and diphenylhydantoin cause a shift in the curve relating T_m to membrane potential, but do not alter the T_h curve. Further, both drugs are capable of inhibiting the shifts in both the T_h and T_m curves

normally produced by reduction of the external calcium concentration to .36 mM. In the case of diphenylhydantoin the effects on T_m and T_h in reduced external calcium were observed at a drug concentration which had no effects in 1.8 mM calcium. This was not observed in the case of phenobarbital, but this might have been due to insufficient testing with different concentrations of phenobarbital.

Since adenosine triphosphate can block spontaneously firing nerves and in addition is known to form a complex with calcium (Abood, 1969), it has been proposed by Seeman (1972) that "negative anesthetics" such as diphenylhydantoin and phenobarbital might block spontaneously firing of axons in a manner similiar to that of ATP. That is, by forming crosslinks between the membrane and calcium they could electrically stabilize the membrane. The shifts in the curves of peak sodium conductance versus membrane potential and the slowing of the time to peak of the sodium current observed with pentobarbital could be explained in a similar manner (Blaustein, 1968). It was postulated that the charged part of the pentobarbital molecule might bind calcium, whereas the lipid soluble portion of the molecule would dissolve in the membrane lipids. The increased surface concentration of calcium would then produce the shifts in kinetic parameters similar to those produced when the

calcium concentration is increased.

There are, however, several difficulties with this explanation. First, pentobarbital does not appear to increase the binding of calcium to membranes (Seeman et al, 1971). Second, adenosine monophosphate and diphosphate are also able to block spontaneous activity and yet are not very effective at complexing calcium (Kuperman et al, 1967). Third, if diphenylhydantoin and phenobarbital were to increase the degree of calcium binding near the external surface of the membrane, then one would expect to see alteration in the curves of both T_m and T_h, as occurs with elevated calcium concentrations rather than in the case of T_m alone. In the experiments reported in this thesis only T_m was altered by phenobarbital and diphenylhydantoin in 1.8 mM calcium Ringer. Thus it would appear rather unlikely that an increase in calcium binding is the mechanism responsible for the abolition of spontaneous firing by these drugs.

One possible experiment which might help to clarify the role of calcium in the mechanism of action of the antiepileptic drugs would be to test these drugs on axons which have been induced to fire repetitively by small, prolonged depolarizations, rather than reduction of the calcium concentration. By this procedure, the extracellular calcium concentration would not have to be

altered and thus the effects of the drugs would not be confused by the effects produced by changing the calcium concentration.

Calcium-Narcotic Interactions

As mentioned in Chapter 2, the sites on the membrane which bind calcium have been implicated as the same sites where narcotics act to block excitability. Although these calcium binding sites might influence the action of certain cationic drugs such as procaine, my experiments generally failed to show any significant effect of altered calcium concentration on the ability of the different drugs tested to reduce $\bar{g}_{Na}$ or $\bar{g}_{K}$. The one possible exception to this was the single experiment with chlorpromazine in which the ability of chlorpromazine to reduce $\bar{g}_{Na}$ and $\bar{g}_{K}$ was enhanced by lowering the calcium concentration from 1.8 to .36 mM. The recovery of the nodal currents were so poor, however, that the significance of this observation is difficult to ascertain. It is possible however, that an insufficient concentration of chlorpromazine was employed in the other experiments involving chlorpromazine-calcium interactions.

Strychnine

Strychnine is a potent central nervous system stimulant capable of producing convulsions presumably by blocking direct and indirect inhibition (Esplin et al, 1969). Although strychnine acts on post-synaptic inhibition it remains to be determined whether its site of action is pre-synaptic or post-synaptic. The actions of strychnine in the central nervous system have been studied intensively, but only a few reports have appeared concerning its actions on isolated neurons (Ajmone-Marsan, 1969).

In the experiments reported in this thesis strychnine reduced $\bar{g}_{Na}$ and $\bar{g}_{K}$, prolonged the duration of the action potential, and produced a voltage-dependent inactivation of the potassium system. These findings are in agreement with other reports of the action of strychnine on isolated neurons (Ajmone-Marsan, 1969; Klee et al, 1973; Maruhashi et al, 1956).

Neither T_h, T_m, or T_n was altered by low concentrations (.01 mM) of strychnine, but with higher concentrations (.1 to .5 mM), there must have been some alteration of the sodium system to account for the prolonged action potentials which were produced. Calculations of the current flow and conductance changes

during the nodal action potential have demonstrated that the falling phase of the action potential is due mainly to the "turning-off" of the sodium system (Dodge, 1963; Frankenhaeuser and Huxley, 1964). This explains for example, why toad and frog nodes do not produce prolonged action potentials when exposed to tetraethylammonium (TEA) which reduces $\bar{g}_K$. Reducing $\bar{g}_K$ will prolong the action potential, but only to a small extent (Frankenhaeuser and Huxley, 1964). Therefore, for strychnine to prolong the duration of the action potential in Ringer by two or three fold, there must be a prolonged flow of sodium current.

There are two possible explanations for a prolonged flow of sodium current; 1) the kinetics of sodium inactivation are altered such that the rate of inactivation is slowed or; 2) the kinetics of the inactivation process are normal but the process does not go to completion. Nickel ions prolong action potentials at the node of Ranvier by slowing both the rate at which the sodium system turns on and the rate at which it turns off (Dodge, 1961; Hille, 1968b). DDT (1,1,1-trichloro-2,2-bis (p-chlorophenyl)-ethane), on the other hand, appears to hold sodium channels open without affecting either h or m (Hille, 1968a). My experiments with strychnine do not allow exclusion of either of these explanations. The difficulty in determining how strychnine alters the sodium system is that $\bar{g}_{Na}$ is reduced

to such an extent that reliable measurements become impossible by the techniques employed.

Another interesting feature of the action of strychnine is its ability to produce potassium inactivation. Potassium inactivation has been observed with a variety of drugs including tertiary and quaternary tropine esters, dibucaine, quaternary analogs of lidocaine, veratranine (5-veratranine-3β, 11α-diol), and internally applied quanternary ammonium ions such as TEA (Armstrong, 1969, 1971; Armstrong and Hille, 1972; Blaustein, 1968b; Chta et al, 1973; Narahashi et al, 1969; Koppehofer and Vogel, 1969; Strichartz, 1973).

Quaternary ammonium ions, when applied internally in both squid axons and the frog node, produce potassium inactivation by blocking the potassium channel, but only when the channel is open (Armstrong, 1969, 1971; Armstrong and Hille, 1972). It has been suggested by Armstrong (1969, 1971) that the quaternary ammonium ions are able to fit into the potassium channel but are unable to pass through. As a consequence, when the membrane is depolarized and the potassium gates open, both potassium and the quaternary ammonium ions are driven into the potassium channel, but only the potassium ions are able to pass through. As the depolarization of the membrane proceeds, more of the potassium channels become blocked so

that the potassium current declines.

Whether the inactivation of the potassium system produced by the various quaternary drugs and the veratrum and strychnine alkaloids is achieved in the same manner is not known but it would appear rather unlikely. Since the radius of TEA is the same as that of a potassium ion with one hydration shell it seems likely that TEA can pass into the potassium channel. This certainly does not apply to either strychnine or veratranine.

The fact that strychnine and some of the veratrum alkaloids both produce potassium inactivation and prolong action potentials suggests they may have a similar mechanism of action. Since so little is known regarding the mechanism of action of the 'labilizers' it would seem worthwhile to explore further the action of strychnine. Although this will probably not increase our understanding of the action of strychnine on post-synaptic inhibition it may be useful in clarifying the process of excitation in nerve.

Summary and Ccnclusions

1. Nodes of Ranvier, when exposed to narcotics such as phenobarbital, pentobarbital, chlorpromazine, chloral hydrate, trichloroethanol, procaine and diphenhydramine, show reduced sodium currents. Analysis of the voltage clamp data revealed that the reduced sodium currents were due to a decrease in the maximum sodium conductance.

2. A number of the narcotics reduced the potassium currents by reducing the maximum pctassium conductance. Only trichloroethanol and pentobarbital however, reduced $\bar{g}_K$ to a greater extent than $\bar{g}_{Na}$.

3. Altering the extracellular calcium concentration did not modify the ability of the barbiturates or trichloroethanol tc reduce either the sodium or potassium conductances. The potency of chlorpromazine however, appeared to be increased in the presence of reduced extracellular calcium.

4. Several of the narcctics altered the kinetics of the sodium and potassium systems. Phenobarbital increased the time necessary for the sodium system to 'turn-on' without effecting the pctassium system kinetics, while pentobarbital slowed the 'turning-cn' of the potassium system without effecting the sodium system kinetics.

Further, chloral hydrate and trichloroethanol increased the time necessary for the sodium and potassium systems to 'turn-on' but decreased the time necessary for the sodium system to 'turn-off'.

5. The antiepileptic agents diphenylhydantoin and phenobarbital prolong the 'turning-on' of the sodium system in 1.8 mM calcium Ringer, but prevent or reduce the normal shifts in the sodium time constants seen in .36 mM calcium Ringer.

6. Strychnine reduced both the sodium and potassium currents and produced potassium inactivation. Neither T_h, T_m, or T_n were effected by strychnine, but at higher drug concentrations where the kinetics were not measured there must have been some modification of the sodium system as the duration of the action potential was prolonged two to three fold.

7. The conclusions reached are as follows:

a) There is probably no single mechanism by which $\bar{g}_{Na}$ is reduced by all drugs.

b) The activation of the sodium system, inactivation of the sodium system, and activation of the potassium system are distinct processes.

c) Stabilization of hyperexcititable membranes by diphenylhydantoin and phenobarbital is related to the

ability of these drugs to prevent changes in sodium activation brought about by conditions leading to hyperexcitablity such as reducing the extracellular calcium concentration.

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